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# A CONFORMATIONAL ANALYSIS FOR LEUCINE-ENKEPHALIN USING ACTIVITY AND BINDING DATA OF SYNTHETIC ANALOGUES

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**1** Leucine-enkephalin and some analogues were assayed for activity *in vitro* on the mouse vas deferens and for binding to opiate receptors from rat brain.

**2** The experimental data were analysed in terms of the stringency for glycine, a D-amino acid or an L-amino acid at each position in the peptide.

**3** The observed configurational specificity was compared with the stringency that would be predicted to occur if enkephalin adopted certain hydrogen-bonded conformations at the receptor.

**4** A small subset of the conformations examined was found to be compatible with the experimental data.

## Introduction

Many procedures have been used to investigate peptide conformations in solution, including nuclear magnetic resonance spectroscopy, optical rotation and circular dichroism studies, and the calculation of internal energies associated with various conformations. Seldom is a conformation uniquely indicated by these methods for short or medium length peptides. Evidence pertaining to peptide conformation at the receptor has invariably been indirect since a pure peptide-receptor complex has not so far been obtained. Marshall & Bosshard (1972) have advocated the synthesis of analogues of biologically active peptides with various residues substituted by amino acids such as proline and dimethyl glycine which exhibit restricted conformational freedom. A study of the activity of these peptides may then be correlated with the range of conformations open to each and the range of plausible conformations becomes reduced. In our studies on leucine-enkephalin and its analogues, we also have chosen to examine *in vitro* biological activity data, and in addition receptor binding data, obtained with preparations of rat brain which have high opiate receptor content.

The enkephalins from pig brain were isolated and sequenced by Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris (1975) and Kosterlitz & Hughes (1975), and shown to have the structures leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) and methionine-enkephalin (Tyr-Gly-Gly-Phe-Met). Their opiate-like effects on innervated smooth muscle preparations were demonstrated. In pig brain, methionine-enkephalin is the preponderant species, but the reverse is true in bovine brain (Simantov & Snyder, 1976). The verification with purified preparations, that the peptides interact at the opiate receptor, combined with our own observations and those of other workers (Bradbury, Smyth & Snell, 1976) showing that conformations are possible for the peptide which could be said to resemble the structures of opiates, makes a more detailed conformational study of the enkephalins of particular interest.

## Methods

### *Vas deferens assay*

The peptides were examined for their ability to inhibit neurally evoked contractions of the isolated vas deferens preparation of the mouse (Hughes, 1975). The relationship between contraction height and concentration was found to be adequately represented by the following equation (Young, 1976):

$$Y = \frac{C + L \cdot Q \cdot X}{1 + Q \cdot X}$$

where $Y$ = contraction (mm), $X$ = dose, $C$ = contraction of tissue at zero dose, $L$ = asymptotic contraction of tissue at infinite dose and $Q$ = potency factor ($= 1/IC_{50}$ M).

In this way, the $IC_{50}$ M, defined as the dose giving the response that is half way between the contraction

of the tissue at zero dose and the asymptotic contraction of the tissue at infinite dose, was calculated. The activity data are presented in Table 1.

*Receptor binding assay*

Whole rat brain was homogenized at 4°C in 50 mM Tris HCl buffer (pH 7.4) in a Polytron homogenizer. The homogenate was then centrifuged at 45,000 *g* for 20 minutes. The supernatant was discarded and the pellet resuspended in 50 mM Tris HCl buffer (pH 7.4). Aliquots (2 ml) of this preparation were then incubated with [$^3$H]-naloxone (Radiochemical Centre, Amersham 21.3 Ci/mol) 30,000 ct/min and test substances at 0°C for 3 h with 100 µg/ml of bacitracin. The reactants were then filtered on Whatman GFB glass fibre pads under vacuum and washed with 10 ml of ice cold incubation buffer. The filter pads were then placed in scintillation vials with sodium dodecylsulphate (1 ml of 1% solution) overnight. Bray's scintillant (10 ml) was then added and the radioactivity determined by scintillation counting. The $IC_{50}$ M (see Table 1) was the concentration of test compound which halved naloxone binding. The reciprocal of the $IC_{50}$ M is taken as a measure of affinity.

*Conformational analysis*

The procedure we have used to examine enkephalin conformation is related to one previously mentioned by Monahan, Amoss, Anderson & Vale (1973). In their study of luteinizing hormone releasing hormone, a type II $\beta$-bend conformation was proposed when it was found that replacement of an internal glycine residue by a D-amino acid promoted activity, and conversely that replacement by the corresponding L-residue reduced activity. However, the conformation assigned was only one of several possibilities, as it is known that a number of conformations can show specificity for D- or for L-residues. We have examined the configurational specificity in the binding and activity data on enkephalin analogues and explored the restrictions on conformational space thereby imposed, in a way related to that of Marshall & Bosshard (1972), but without the use of residues such as dimethyl glycine (related to L- and D-alanine) which may be regarded as containing a side-chain composed of both the L- and the D-configurations of the parent amino acid. We have restricted this presentation to some particularly stable conformations of the peptide chain which are stabilized by intramolecular hydrogen bonds, and which have been described previously.

The various conformations can be described in terms of the torsion angles of each bond in the peptide chain, as defined by the IUPAC-IUB Commission (1970). In the present study it has been assumed that the peptide group is planar and *trans* ($\omega = 180°$). *Cis* conformations have been found in cyclic peptides and

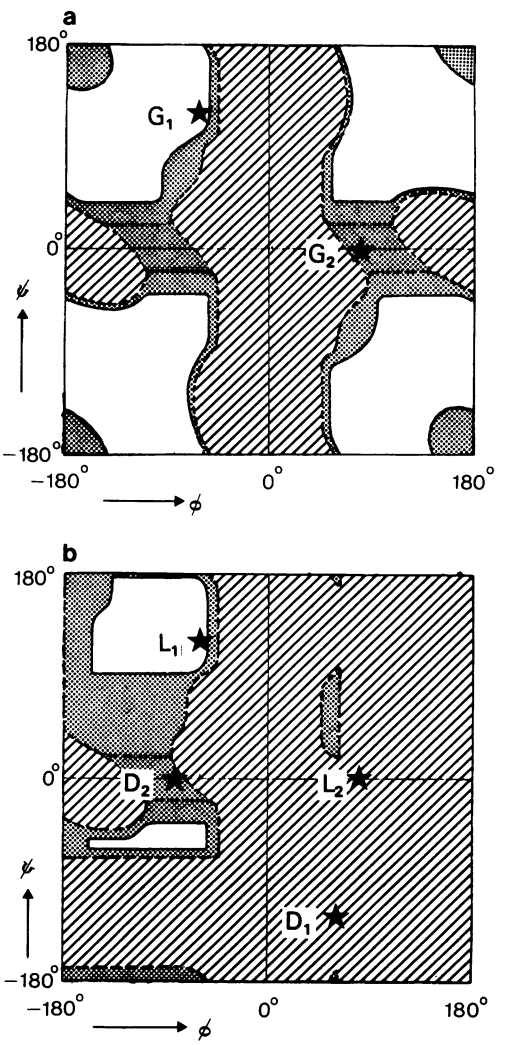


**Figure 1** Conformational maps for (a) glycine and (b) L-alanine. Disallowed combinations of $\phi$ and $\psi$ are hatched. For these, atoms would be too close. Allowed areas are clear. Shaded areas (allowed) correspond to marginally close approaches (e.g. hydrogen bonds). The two residues (1,2) in a type II $\beta$-bend conformation are plotted according to each being glycine (G), an L-amino acid (L) or a D-amino acid (D). Residue 1 may be L (or G) but not D. Residue 2 may be D (or G) but not L. The configurational specificity of the bend is therefore shown in Table 2 as LD.

at proline residues but very rarely in acyclic peptides lacking proline (Ramachandran & Mitra, 1976). Since $\omega$ is restricted, it has been useful to construct a two-dimensional plot of $\phi$ and $\psi$, the torsion angles about N–$C_\alpha$ and $C_\alpha$–C′ respectively, the remaining bonds in the peptide chain (Ramachandran, Ramakrishnan &

Sasisekharan, 1963; Ramakrishnan & Ramachandran, 1965). The bond distances and angles used were those of Corey & Pauling (1953) and allowed contact distances were specified by Ramachandran *et al.* (1963). The regions of accessible conformational space for a glycine and an L-alanine residue are shown in Figure 1a and 1b respectively. The maps for other L-amino acids resemble that for alanine, though some are more restricted.

The conformations we have chosen to study are shown in Table 2. Torsion angles were from Lewis, Momany & Scheraga (1973), Matthews (1972), Némethy & Printz (1972) and Ramakrishnan & Ramachandran (1965). The configurational specificity at each residue position in these conformations was estimated by plotting the $\phi$ and $\psi$ values. Each amino acid residue in a chosen conformation maps at a point in the $\phi,\psi$-plot. A conformation may involve one or several amino acid residues. For example, the type II $\beta$-bend conformation contains two residues and the conformation for each of these is separately plotted in Figure 1. Each residue is plotted according to it being glycine, an L-amino acid or a D-amino acid. A D-residue is plotted on the map for an L-residue by inverting the $\phi$ and $\psi$ angles before plotting. It is seen that the conformation does not allow a D-residue at position 1 or an L-residue at position 2. The configurational specificities of the other conformations in Table 2 were similarly determined including the mirror-image conformations (denoted by primes), and are shown in the Table.

## Results

### *Relationship between activity and binding*

Before examining structure/activity relationships, it was essential to determine the degree of correlation between the two methods of assay used in this study, the vas deferens and the opiate receptor binding assays. The correlation between the log potencies in the vas deferens assay ($y$) and in the receptor binding assay ($x$) defined as $\log_e(1/IC_{50}.M)$ was studied using data for the compounds in Table 1 (excluding those for which no value or only a threshold value for binding is shown in the Table) and gave a correlation coefficient of +0.95.

The regression equations were

$$y = 0.928\ x + 2.10$$

$$x = 0.981\ y - 1.01$$

From these, the slopes $dy/dx$ were 0.928 and 1.019 respectively, indicating a one-to-one correspondence between the two variables. The high correlation obtained was not primarily the result of using a small sample of selected compounds. In a similar analysis for 52 miscellaneous analogues (Beddell *et al.*, 1977) including those in the previous analysis, the correlation coefficient, 0.84, was still high and slopes of the regression (0.842, 1.180 respectively) were still quite close to unity.

### *Conformational analysis*

The top line in Table 2 summarizes the activity and binding data shown in Table 1. The configurations at each position in the peptide which favour activity and binding are shown. For example, at position 1, L-tyrosine favours activity and binding; these are reduced several hundred-fold when D-tyrosine is incorporated. Thus the L- but not the D-configuration is allowed. At position 2 on the other hand, the reverse is the case. Activity and binding are low when L-alanine is present, but high when D-alanine (or glycine) is present, provided that substitutions elsewhere in the molecule do not inhibit activity and binding. At position 3 glycine is strongly preferred to an L- or D-residue. Position 4, like position 1, favours the L-configuration. Finally at position 5, either an L- or D-residue can be accommodated with retention of activity and binding. In the remainder of Table 2 we consider each conformation in turn in each successive possible alignment in the peptide chain and show the configurational specificity appropriate to the conformation. The right hand column of Table 2 indicates agreement or contradiction between the configurational specificity of a conformation in a certain alignment within the peptide and that deduced for enkephalin from activity and binding data. The conformation in a particular alignment is invalid when the activity and binding data allow an L- or D-configuration at a position where such is disallowed by the conformation.

## Discussion

The extent to which estimates of biological activity or receptor binding relate to the interaction between peptide and receptor depends on the extent to which other factors, such as diffusion barriers, peptide metabolism and non-specific binding interfere. The effects of such factors if present, would probably be different in the two assays used here and a strong correlation between the two sets of assay data would imply that interference is minimal. It would seem, from the observed correlation between the two assay methods, that the present data reflect the peptide-receptor interaction itself and are not unduly influenced by other factors.

Table 2 shows the resultant valid conformations for enkephalin. Of these, some such as the helix structures, though not inconsistent with the activity and binding data, explain little or none of the configurational specificity found in them. Three structures, namely the inverse $\gamma$ turn, the V′ turn and

the $\beta$II′ bend could explain the preference for a D-residue in position 2. These and the $\beta$I bend could also account for the preference for an L-residue in position 4. Only the inverse $\gamma$ turn can simultaneously explain both. Finally, no conformation examined can explain the stringency for glycine in position 3. Although the inverse $\gamma$ turn explains the experimental data most completely, it would be premature to conclude that this is the conformation of enkephalin at the receptor. There are respects in which the present analysis could be extended. There is for example disagreement concerning the configurational specificity of some of these conformations, notably the $\gamma$ turn (Bleich, Galardy & Printz, 1972; Lewis *et al.*, 1973). Furthermore the present treatment excludes deviation of $\omega$ from 180°, although it has been well demonstrated that substantial non-planarity of the peptide group can occur. More detailed studies exploring the significance of these factors and extending the range of conformations to include

**Table 1** Activity of enkephalin analogues *in vitro* on mouse vas deferens and binding to rat brain receptors, both estimated as $10^{-7}/IC_{50}$ M

| *Structural formula* | *Activity on vas deferens* ($10^{-7}/IC_{50}$ *M*) | *Receptor binding* ($10^{-7}/IC_{50}$ *M*) |
|---|---|---|
| *Tyr*- Gly- Gly- Phe- Leu | 8.17 | 9.09 |
| D-*Tyr*- Gly- Gly- Phe- Leu | 0.034 | – |
| *Tyr*-D-Ala- Gly- Phe- Leu | 57.3 | 31.3 |
| D-*Tyr*-D-Ala- Gly- Phe- Leu | 0.15 | 0.051 |
| *Tyr*-D-Ala- Gly- Phe-D-Leu | 263.0 | 38.5 |
| D-*Tyr*-D-Ala- Gly- Phe-D-Leu | 0.064 | <0.010 |
| Tyr- *Gly*- Gly- Phe- Leu | 8.17 | 9.09 |
| Tyr- *Ala*- Gly- Phe- Leu | 0.006 | 0.020 |
| Tyr-D-*Ala*- Gly Phe- Leu | 57.3 | 31.3 |
| Tyr- *Gly*-D-Ala- Phe- Leu | 0.021 | 0.002 |
| Tyr- *Ala*-D-Ala- Phe- Leu | 0.009 | <0.010 |
| Tyr-D-*Ala*-D-Ala- Phe- Leu | 0.075 | 0.026 |
| Tyr- *Gly*- Ala- Phe- Leu | 0.18 | 0.011 |
| Tyr- *Ala*- Ala- Phe- Leu | 0.009 | <0.010 |
| Tyr-D-*Ala*- Ala- Phe- Leu | 2.58 | 1.26 |
| Tyr- Gly- *Gly*- Phe- Leu | 8.17 | 9.09 |
| Tyr- Gly- *Ala*- Phe- Leu | 0.18 | 0.011 |
| Tyr- Gly-D-*Ala*- Phe- Leu | 0.021 | 0.002 |
| Tyr-D-Ala- *Gly*- Phe- Leu | 57.3 | 31.3 |
| Tyr-D-Ala- *Ala*- Phe- Leu | 2.50 | 1.26 |
| Tyr-D-Ala-D-*Ala*- Phe- Leu | 0.075 | 0.026 |
| Tyr- Ala- *Gly*- Phe- Leu | 0.006 | 0.02 |
| Tyr- Ala- *Ala*- Phe- Leu | 0.009 | <0.010 |
| Tyr- Ala-D-*Ala*- Phe- Leu | 0.009 | <0.010 |
| Tyr- Gly- Gly- *Phe*- Leu | 8.17 | 9.09 |
| Tyr- Gly- Gly-D-*Phe*- Leu | 0.002 | <0.011 |
| Tyr-D-Ala- Gly- *Phe*- Leu | 57.3 | 31.3 |
| Tyr-D-Ala- Gly-D-*Phe*- Leu | 0.043 | 0.011 |
| Tyr- Gly- Gly- Phe- *Leu* | 8.17 | 9.09 |
| Tyr- Gly- Gly- Phe-D-*Leu* | 9.66 | 4.00 |
| Tyr-D-Ala- Gly- Phe- *Leu* | 57.3 | 31.3 |
| Tyr-D-Ala- Gly- Phe-D-*Leu* | 263.0 | 38.5 |
| Morphine | 0.215 | 28.6 |

The first compound is leucine-enkephalin. Data for 16 analogues of leucine-enkephalin are given and the compounds are arranged in pairs or triplets to facilitate comparison of a D for L substitution or of a D or L for glycine substitution respectively.

alternative hydrogen-bonds, should be useful and some non-hydrogen-bonded conformations. Despite the limitations of the present analysis, it is of interest to compare the conformations proposed for enkephalin by other workers with those in Table 2. Bradbury *et al.* (1976) have advocated a $\beta$ bend at positions 2 and 3 from studies of $\beta$-lipotropin and structural comparison with opiates. Jones, Gibbons & Garsky (1976) and Roques, Garbay-Jaureguiberry, Oberlin, Anteunis & Lala (1976) have advocated a $\beta$I turn in positions 3 and 4 from proton magnetic resonance data on enkephalin solutions. Neither of these suggested conformations would be excluded by our studies. Isogai, Némethy & Scheraga (1977) have calculated that the conformation of minimum energy is a type II′ $\beta$-bend in positions 3 and 4, also shown as possible for the receptor-bound peptide in Table 2. De Coen, Humblet & Koch (1977) have described 15 low energy conformations for enkephalin, including a type II′ $\beta$-bend at positions 2 and 3, a type II $\beta$-bend at positions 3 and 4, a type I $\beta$-bend in position 3 and 4, and type V and type V′ bends at positions 2 and 3. Only some of these are valid in Table 2 for receptor-bound enkephalin.

**Table 2** Comparison between the observed configurational specificity of enkephalin (top line) and that predicted if enkephalin were to adopt certain named hydrogen-bonded conformations

| | *Position* | | | | | |
|---|---|---|---|---|---|---|
| *Structure* | *1* | *2* | *3* | *4* | *5* | *Validity* |
| Enkephalin | L | D | G | L | A | |
| $\gamma$-turn | *G*(L) | D | L | | | × (?) |
| | | *G*(L) | D | L | | × |
| | | | G(L) | *D* | *L* | × |
| $\gamma'$-turn | *G(D)* | *L* | D | | | × |
| | | *G*(D) | L | *D* | | × |
| | | | G(D) | L | *D* | × |
| Inverse $\gamma$ turn | | D(L) | L | L | | √ |
| Inverse $\gamma'$ turn | | *L*(D) | D | *D* | | × |
| V turn | | *L* | D | | | × |
| | | | L | *D* | | × |
| V′ turn | | D | L | | | √ |
| | | | D | L | | √ |
| $\beta$I bend | | A | L | | | √ |
| | | | A | L | | √ |
| $\beta$I′ bend | | A | D | | | √ |
| | | | A | *D* | | × |
| $\beta$II bend | | *L* | D | | | × |
| | | | L | *D* | | × |
| $\beta$II′ bend | | D | L | | | √ |
| | | | D | L | | √ |
| $\beta$III or III′ bend | | A | A | | | √ |
| | | | A | A | | √ |
| $2_7,2.2_7^R$ helices | L | *L* | L | L | *L* | × |
| $2_7',2.2_7^L$ helices | *D* | D | D | *D* | *D* | × |
| $\gamma^R$ helix, $\gamma^L$ helix | *G* | *G* | G | *G* | *G* | × |
| $3_{10}^{R,L}$ or $\alpha^{R,L}$ helices | A | A | A | A | A | √ |

Each possible alignment within the peptide of the various conformations is shown. G, L, D, A respectively indicate positions which accept glycine only, an L-residue, a D-residue and both L- and D-residues. Italics are used to denote points in the peptide where the observed and predicted configurational specificities are in conflict. The disagreement is shown by a cross in the right hand column. Some points on the $\phi, \psi$ plot are within 15° of an allowed area, and distortions of peptide geometry might conceivably place these in the allowed area. The configurational specificity that would result from this is shown in parentheses.

The analytical procedures described for enkephalin in the present study are of general applicability to peptides, provided appropriate assays are available. In the present study, more than half of the starting set of conformations was eliminated. This significant clarification was possible because the activity data were well behaved, substitutions producing either substantial or small, but not in general intermediate levels of effect. For other peptides this may not always be the case. The method described here is well suited to discrimination between a starting set of low energy peptide conformations. It is not necessary that these should be hydrogen-bonded, as in the present study. Indeed De Coen *et al.* (1977) have proposed some additional non-hydrogen bonded conformations for enkephalin which merit further study. Provided that a set of low-energy peptide conformations can be obtained from a variety of methods such as nuclear magnetic resonance, model-building, crystallography and energy calculation, the study of configurational specificity in binding and activity data could be used to select those conformations which the peptide might adopt at the receptor.

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