Conformation of [Leu^5]Enkephalin from X-ray Diffraction: Features Important for Recognition at Opiate Receptor

Abstract. *The conformation of [Leu^5]enkephalin is produced by a Tyr-Gly-Gly-Phe β bend stabilized by antiparallel hydrogen bonding between tyrosine and phenylalanine. On the basis of a comparison of the observed structure with the structure of known opiate agonists, three hydrophilic and two hydrophobic regions have been identified as contributing to the recognition of the molecule at the opiate receptor site.*

Enkephalin, the endogenous morphine-like substance first isolated by Hughes from pig brain (*1*), is a mixture of two pentapeptides, H-Tyr-Gly-Gly-Phe-Met-OH ([Met^5]enkephalin) and H-Tyr-Gly-Gly-Phe-Leu-OH ([Leu^5]enkephalin) (Fig. 1a) (*2*). The ratio of [Met^5]enkephalin to [Leu^5]enkephalin appears to be species-specific; pig brain contains four times more of the former than the latter (*1*), whereas in cattle brain the ratio is reversed (*3*). The enkephalins bind to the opiate receptor in brain, spinal cord, and gut and displace naloxone, a powerful opiate antagonist, from that site.

Prior to the isolation of enkephalin, Pert and Snyder developed a simple, sensitive assay for opiate receptor binding by which they could predict the extent to which a drug is an opiate agonist, antagonist, or mixed agonist-antagonist (*4*). Thus, the structural features of a large number of opiate drugs could be correlated with their binding to the opiate receptor and mode of action (*5*). 7-[1-Phenyl-3-hydroxybutyl-3-]endoethenotetrahydrothebaine (PET) (Fig. 1c), a derivative of morphine (Fig. 1b) and a much more potent opiate agonist than the parent drug, contains the features common to the most powerful opiates: a phenol ring (A ring), an amine nitrogen group, and a phenyl ring (F ring) in a precisely defined three-dimensional pattern (*5–7*). Because of the existence of rigid opiate agonists, there has been a great deal of speculation on what conformation the more flexible enkephalin molecule assumes in order to bind to the same receptor (*8–13*). We now report the determination of the molecular conformation of [Leu^5]enkephalin in the solid state by single crystal x-ray diffraction.

Small, thin crystals of [Leu^5]enkephalin, $C_{28}H_{37}O_7N_5 \cdot H_2O$, were grown from an aqueous methanol solution. They crystallize as the monohydrate in the monoclinic space group $C2$ and have a calculated density of 1.13 g cm^{-3}; the cell constants are a = 31.871(6) Å, b = 8.535(2) Å, c = 12.467(2) Å, β = 96.53(2)°, and Z = 4. With the use of nickel-filtered copper radiation, 2676 independent intensities (sin θ_{max}/λ = 0.562 Å^{-1}, where θ is the Bragg angle and λ the wavelength) were measured with an Enraf-Nonius CAD-4 diffractometer. A total of 1132 reflections were found to have intensities greater than two times their standard deviations and were therefore considered observed; the remaining 1544 intensities were coded as unobserved and not used in the refinement. The structure was solved by means of the direct methods program QTAN (*14*). During the initial stages of

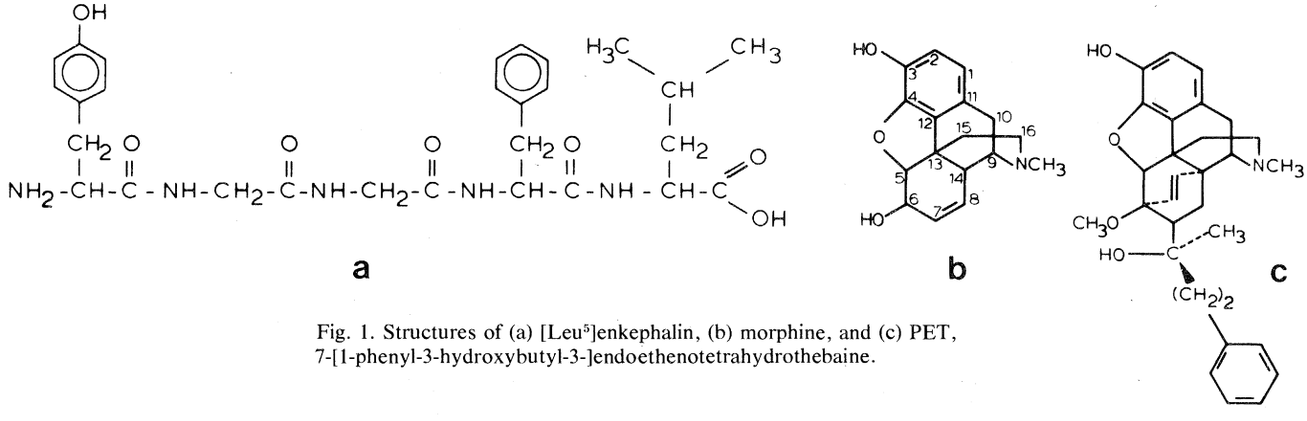

Fig. 1. Structures of (a) [Leu^5]enkephalin, (b) morphine, and (c) PET, 7-[1-phenyl-3-hydroxybutyl-3-]endoethenotetrahydrothebaine.

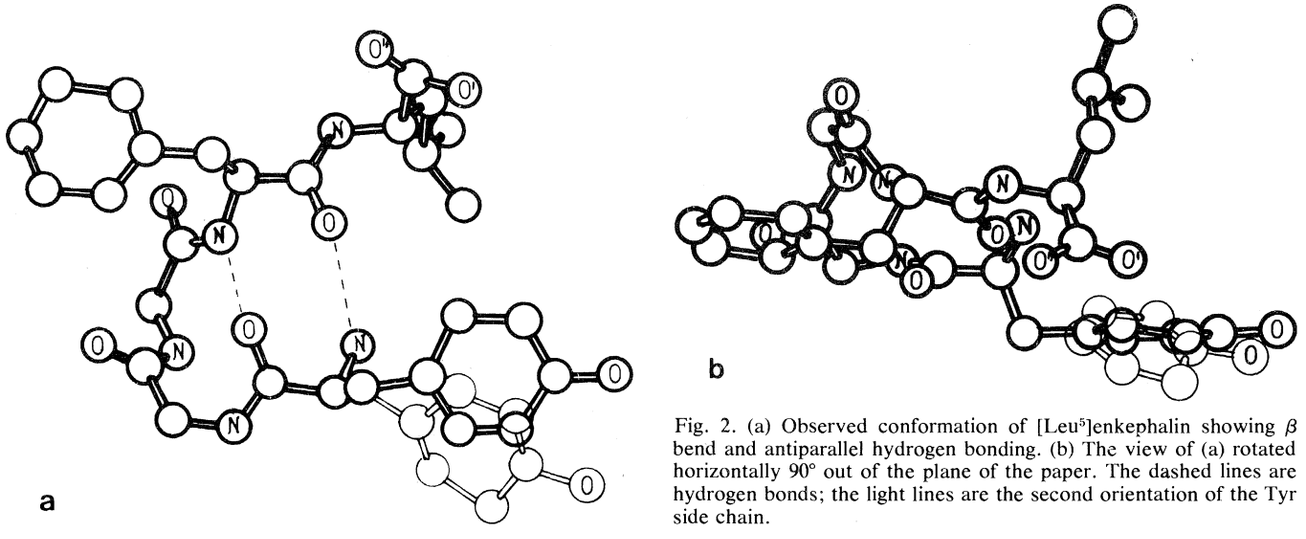

Fig. 2. (a) Observed conformation of [Leu^5]enkephalin showing β bend and antiparallel hydrogen bonding. (b) The view of (a) rotated horizontally 90° out of the plane of the paper. The dashed lines are hydrogen bonds; the light lines are the second orientation of the Tyr side chain.

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Fourier refinement, it was found that the phenol ring of the Tyr residue is disordered and occupies two discrete positions with nearly equal frequency. The structure has been refined by full-matrix least-squares to a residual of 0.14; the vibration of all atoms was treated isotropically. Because of the limited number of observed data, a result of the size of the crystal and the disordered Tyr side chain, it was not possible to locate hydrogen atoms.

The observed conformation of [Leu^5]enkephalin is shown in Fig. 2 and includes both orientations of the disordered Tyr side chain. Two intramolecular hydrogen bonds (Fig. 2a, dashed lines) produce a β bend in the sequence Tyr-Gly-Gly-Phe. These bonds occur between the amino nitrogen of Tyr and the carbonyl oxygen of Phe (2.86 Å) as well as between the amino nitrogen of Phe and the carbonyl oxygen of Tyr (2.99 Å). The observed conformation orients all side chains in exposed positions on the surface of the molecule. If the backbone of the peptide is thought of as approximating a plane, the Tyr and Phe side chains are located on the same side of this plane, and the side chain of Leu is on the other side of this hypothetical plane and directed nearly perpendicularly away from it (Fig. 2b). The carbonyl groups of both Gly residues are directed away from the internal bend of the molecule. All peptide linkages are *trans*; the ϕ, ψ, and χ torsion angles are listed in Table 1.

All potential hydrogen bond donors and acceptors, with two exceptions, are involved in intermolecular hydrogen bonds to either a symmetry related enkephalin molecule or to the water molecule. The two exceptions are N(4) and O(1) which produce the inner intramolecular hydrogen bond in the β bend. While the remaining nitrogen and oxygen atoms are involved in at least one intermolecular hydrogen bond, the amino group of Tyr residue, the hydroxyl group of the Tyr residue, and the carboxyl group of the Leu are involved in two or more hydrogen bonds. The observed hydrophilicity of these three groups in the crystal structure suggests that these atoms are the ones likely to be involved in hydrogen bonding to the receptor. A large hydrophobic region is also observed in the crystal structure and involves the Phe and Leu side chains of adjacent molecules.

As a result of their studies of solutions, two groups have predicted a folded conformation with a β bend and a hydrogen bond between the amino group of Met^5 and the carbonyl of Gly^2 (*8*); a third study suggested that the molecules are inflexible on a chemical time shift scale because of the nonequivalence of the α protons of Gly^2 and Gly^3 in both H_2O and dimethyl sulfoxide (*9*). However, results of Kahled and co-workers, who used a combination of circular dichroism, ultraviolet, and ^{13}C nuclear magnetic resonance (NMR) spectra at varying concentrations, cast some doubt on the conclusions of the previous studies (*10*). Kahled *et al.* concluded that dimers and higher aggregates are present at the concentrations normally used for NMR studies and, therefore, may have confused the assignment of peaks.

Table 1. Torsion angles for amino acid residues.

	(1) Tyr	(2) Gly	(3) Gly	(4) Phe	(5) Leu
ϕ		59	97	−136	−105
ψ	126	25	−7	145	−4
ω	173	179	−174	180	
χ^1	$\begin{pmatrix}-43\\-86\end{pmatrix}$			−62	−69
$\chi^{2,1}$	$\begin{pmatrix}-89\\-30\end{pmatrix}$			90	178

Four theoretical studies have also attempted to predict conformation (*11*, *12*). The inner hydrogen bond (between the Phe amino and the Tyr carbonyl) and the crystallographically observed β bend were predicted by Bradbury *et al.* (*12*) from the application of the empirical rules for predicting secondary structure from amino acid sequence. The presence of the two hydrogen bonds observed in our study has never been proposed, but must contribute to a reduction of the total energy of the system and the stabilization of the observed backbone conformation.

Attempts to account for the similar mode of action of the various opiates and that of enkephalin have generally been based on the premise that at the receptor the Tyr side chain of enkephalin adopts the conformation observed for the "tyramine" moiety of the rigid morphine molecule (*13*). However, the observed tyramine conformation in morphine (*15*) is not that observed for either of the disordered Tyr side chains of enkephalin and furthermore, if the tyramine conformation is imposed on the Tyr side chain of enkephalin, the remainder of the molecule is directed away from any region that would be occupied by morphine at the receptor. Repositioning of the remainder of the enkephalin molecule requires major conformational changes in the peptide backbone accompanied by disruption of the intramolecular hydrogen bonds. We conclude that the relative placement of the nitrogen atom with respect to the hydroxyl group of the Tyr residue is the important feature in enkephalin, not adoption of the conformation observed in morphine.

When an attempt is made to superimpose morphine and enkephalin, a good fit is obtained between the 6-hydroxyl and the amino nitrogen of morphine with the Leu carboxyl oxygen and Tyr amino nitrogen of the enkephalin. This fit brings the Leu side chain into the same region as the C(7)–C(8) region of morphine. However, with this fit the two phenoxy oxygens are approximately 3.6 Å apart (*16*). We conclude that neither of the observed solid state conformations of the Tyr side chain is that required by the receptor. In fact, rotations about the C(α)–C(β) and C(β)–C(γ) bonds of all three side chains are not restricted by the backbone conformation, and in solution these may well take on a number of orientations without disturbing the observed folding of the molecule. Substitution of Met for Leu would also appear to have little effect on the overall conformation, but could account for its difference in activity or activity at receptors in different tissues.

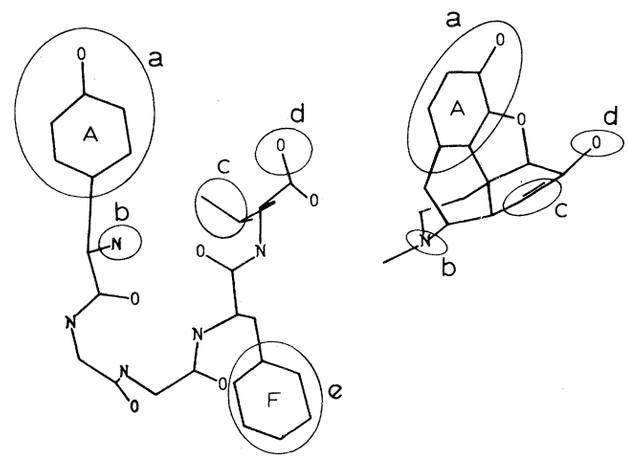

Fig. 3. Stick drawings from crystallographic coordinates of [Leu^5]-enkephalin (this study) and morphine (*15*) illustrating analogous regions. For explanation of (a) to (e) see text.

Previous attempts to delineate features important for binding at the opiate receptor (*5–7*) have been based primarily on the effect of substitution on activity. Our study provides structural data that can be used as a basis for understanding the activity of various analogs. For example, the observed ϕ and ψ torsion angles of both Gly residues describe a conformation that is forbidden to L-amino acids, and therefore the activity of the D-Ala^2 analog (*7, 17*) should not be unexpected since this amino acid can easily adopt the conformation of Gly^2 and at the same time provide protection against proteolysis. The activity of the *N*-methyl-Tyr^1 (*7*) analog can also be understood on the basis of the solid state results since the primary amino group has sufficient space around it for one or two methyl groups without disrupting either the hydrogen bonding scheme or the overall conformation. However, the active D-Ala^2, *N*-methyl-Phe^4 (*17*) analog must have a different conformation from that observed in our study because of the bulk of the additional methyl group and the loss of one of the hydrogen bonds.

Other investigations have enumerated chemical features important for activity at the opiate receptor (*5–7, 17*). Our study provides a rational basis for the delineation of five regions (Fig. 3) which are important, but not all necessary, for opiate activity: (i) a phenyl hydroxyl group, attached to the A ring of morphine, analogous to the Tyr hydroxyl of enkephalin; (ii) an amino nitrogen, the tertiary nitrogen of morphine, analogous to the primary amino group of enkephalin [H-Tyr]; (iii) a hydrophobic region, the C(7)–C(8) face of morphine located in the same region as the Leu side chain and analogous Met side chain in [Met^5]enkephalin; (iv) a hydrophilic group in the region of the O(6) of morphine, which corresponds to the carboxyl terminus of enkephalin; and (v) a phenyl ring, which is missing in morphine but identified as the F ring in a number of more potent opiate drugs, and which corresponds to the relatively unconstrained Phe side chain of enkephalin.

Thus, the peptide backbone of enkephalin stabilized by two intramolecular hydrogen bonds provides the rigid frame to which the side chains are attached in a specific spatial relationship. Optimal binding to the opiate receptor is accomplished by minor adjustments to the orientations of these side chains by rotations about the C(α)–C(β) and C(β)–C(γ) bonds.

G. DAVID SMITH, JANE F. GRIFFIN
Medical Foundation of Buffalo, Inc., 73 High Street, Buffalo, New York 14203

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18. Figures 2 and 3 were drawn on PROPHET, an NIH-sponsored biomedical computer network. We thank D. Rohrer for the supervision of data collection. Coordinates are available from G.D.S. or J.F.G. on request. Supported by PHS grant GM-19684.

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Pupillometric Signs of Brain Activation Vary with Level of Cognitive Processing

Abstract. *The idea that hierarchically higher brain processes require greater amounts of central nervous system vigilance or activation for their execution was tested in two experiments measuring pupillary dilation during the decision interval of a hierarchically structured letter-matching task. Larger dilations indicative of increased activation were observed for letter pairs requiring higher levels of processing.*

Hughlings Jackson (*1*) in 1884 proposed that functional processes in the human nervous system are hierarchically organized, with the higher levels being increasingly unconstrained or plastic, complex, and voluntary as opposed to automatic. Jackson recognized that factors that reduce central nervous system (CNS) vigilance selectively affect the highest levels of integration, an idea which Head later extended in his writings (*2*). By vigilance was meant the general state of nervous system activation that is now thought to be reflected as electrocortical desynchronization and autonomic arousal (*3*).

These early investigations studied the level of integration that may be accomplished when the capacity of the nervous system to sustain a normal state of activation has been impaired by either injury, disease, or the effects of drugs (*2*). A modern example of this experimental approach is the discovery that the aphagia and adipsia following lateral hypothalamic lesions are due in large part to a disruption of endogenous activation systems and that with recovery of these systems the hierarchically organized processes governing feeding and drinking return in a Jacksonian sequence of increasing complexity (*4*). However, it appears that the level of activation in the normal organism is not fixed, but varies from moment to moment and task to task according to the processing demands placed upon the nervous system (*5*).

We now report the results of two experiments that suggest that hierarchically organized cognitive processes vary in the degree to which CNS activation is mobilized during their execution. The cognitive task employed was letter matching, in which a pair of visually presented upper case letters, lower case letters, or one of each are judged by an observer to be the same or different (*6*). If a name criterion is employed as in the first experiment, letter pairs may be judged to be the same if they are orthographically identical (for example, AA or aa) or differ in orthography but share the same name (for example, Aa). Only the physical features of the former pair need be processed before a judgment can be reached, whereas the stimuli must be processed at the higher level of naming for the latter type of pair. When a category criterion is used, as in the second experiment, letters are judged same if they belong to a common category, vowels or consonants. In this case a third

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