SYNTHESIS OF LEUCINE ENKEPHALIN DERIVATIVES: STRUCTURE-FUNCTION STUDIES

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SUMMARY: The solid phase synthesis of highly purified [Leu^5]enkephalin and of seven derivatives including [Ala^2,Leu^5]-, [Ser^2,Leu^5]-, [Ser^3,Leu^5]-, [Aba^2, Leu^5]-, and [des-Gly$^{2(3)}$,Leu^5]enkephalins are reported, and their morphine-like activities in neuroblastoma x glioma cell homogenates were measured. Changes at the 2, 3, and 5 positions of the enkephalin provided analogues which were all less active than [Leu^5]enkephalin. The results are discussed in terms of recently suggested conformational structures for the enkephalin peptides. No melanocyte stimulating activity was observed for [Leu^5]enkephalin, [Ala^2,Leu^5]enkephalin, or [Ser^2, Leu^5]enkephalin.

Recently Hughes et al.(1) have isolated two morphine-like pentapeptides from pig brain, Tyr-Gly-Gly-Phe-Met (methionine enkephalin, [Met^5]enkephalin), and Tyr-Gly-Gly-Phe-Leu (leucine enkephalin, [Leu^5]enkephalin).[2] These peptides bind to a variety of opiate receptors (1-11), and also have been reported to possess melanocyte stimulating hormone-like (MSH-like) activity (12).

Two basic proposals regarding the receptor conformation of opiate-like peptides have been made. The first suggestion was that an α-helix structure (13) could obtain for morphine-like activity for a peptide. At present there is no biological, chemical or physical evidence in support of this suggestion. A later report (14) suggested that a 1→4 turn (β-turn) structure might account for the morphine-like activity of enkephalin. In this β-turn structure, a hydrogen bond between the tyrosine-1 carbonyl oxygen and the phenylalanine-4 peptide amide proton

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[2]Standard abbreviations for amino acids, protecting groups, and peptides are used [J. Biol. Chem., 247, 977 (1972)]. Other abbreviations include: n-BuOH, 1-butanol; n-AmOH, 1-pentanol; Pyr, pyridine; Aba, α-aminobutyric acid.

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was suggested. Very recently, two proton magnetic resonance studies (15,16) have provided evidence that in $^{2}H_{6}$-DMSO solutions, [Met^{5}]enkephalin, and [Leu^{5}]enkephalin may have β-turn (1→4 turn) structures, but that the hydrogen bond involved the glycine-2 carbonyl oxygen and the methionine-5 peptide amide hydrogen. On the other hand, more extensive preliminary ^{1}H and ^{13}C NMR studies (17) found no evidence for a β-turn structure. We report here the synthesis and biological activities of [Leu^{5}]enkephalin and seven analogues of [Leu^{5}]enkephalin designed to test some aspects of these models, and which also further investigate the purported melanocyte stimulating (MSH-like) activity of these compounds.

MATERIALS AND METHODS

Peptide synthesis. Amino acid analyses were performed on a Beckman Model 120-C Automatic Amino Acid Analyzer, after 22 hr hydrolysis of peptides in deaerated 6 N HCl. Thin layer chromatography (TLC) was carried out on Silica-Gel glass plates with use of the following solvent systems: A, n-BuOH:HOAc:H_2O (10:30:25); B, n-BuOH:HOAc:H_2O (4:1:5); C, n-BuOH:HOAc:H_2O (2:1:1); D, Pyr:HOAc:H_2O (50:30:15); E, n-BuOH:HOAc:H_2O:Pyr (15:3:12:10); F, n-AmOH:Pyr:H_2O (35:35:30).

Peptides were synthesized by the solid phase method (18) as used in our laboratory (19). A chloromethylated polystyrene resin-1% cross-linked with divinylbenzene (10g) was substituted to a level of 0.30 mmol Leu/g resin in ethyl acetate. A 3 mole excess of protected amino acid and dicyclohexylcarbodiimide (DCC) was used in a 180 min. coupling reaction for adding each amino acid residue to the growing peptide chain. The synthesis program for addition of each amino acid residue was as follows: [1] CH_2Cl_2 - 1 min x 3; [2] trifluoroacetic acid (25% in CH_2Cl_2) with 2% anisole - 2 min and 20 min; [3] CH_2Cl_2 - 1 min x 3; [4] diisopropylethylamine (10% in CH_2Cl_2) - 2 min x 2; [5] CH_2Cl_2 - 1 min x 4; [6] coupling step of amino acid derivative; [7] CH_2Cl_2 - 1 min x 3; [8] EtOH - 1 min x 3.

The protected peptide resin (0.5 - 1 g) was stirred in 15 ml of HF containing 1.5 ml of anisole for 1 hr at 0°C. After removal of the HF and anisole in vacuo, the peptide was extracted with 3 x 10 ml portions of 30% HOAc, diluted with H_2O and lyophilized. The crude peptide powder was purified by one or two gel filtration on a Sephadex G-15 column (2.2 x 98 cm) using 30% acetic acid. Yields were based on the leucine substitution on the resin.

1. Tyr-Gly-Gly-Phe-Leu ([Leu^{5}]enkephalin) - From Boc-Tyr-Gly-Gly-Phe-Leu-resin in 65% yield. TLC in solvent systems A (R_f 0.77), C (R_f 0.83), D (R_f 0.90), E (R_f 0.63). Amino acid analysis: Tyr 0.99, Gly 2.04, Phe 1.02, Leu 0.96.
2. Tyr-Gly-Gly-Phe-Leu-OMe - Obtained from Boc-Tyr-Gly-Gly-Phe-Leu-resin by methanolysis in presence of catalytic amount of triethylamine in 69% yield. TLC in solvent systems C (R_f 0.80), D (R_f 0.92), E (R_f 0.80). Amino acid analysis Tyr 0.97, Gly 2.01, Phe 1.00, Leu 1.03.
3. Tyr-Gly-Gly-Phe-Leu-NH_2 - Obtained from Boc-Tyr-Gly-Gly-Phe-Leu-resin by ammonolysis in methanol for 45 hrs at room temperature in 66% yield. TLC in solvent systems C (R_f 0.75 and R_f 0.80, 3-5% ester), D (R_f 0.91), E (R_f 0.73). Amino acid analysis: NH_3 1.1, Tyr 0.96, Gly 1.99, Phe 1.0, Leu 1.05.
4. Tyr-Ala-Gly-Phe-Leu - From Boc-Tyr-Ala-Gly-Phe-Leu-resin in 58% yield. TLC in solvent systems B (R_f 0.57), E (R_f 0.68), F (R_f 0.64). Amino acid analysis Tyr 0.97, Ala 0.96, Gly 0.99, Phe 0.99, Leu 1.02.

5. Tyr-Gly-Ser-Phe-Leu - From Boc-Tyr(OBzl)-Gly-Ser(OBzl)-Phe-Leu-resin in 60% yield. TLC in solvent systems B (R_f 0.84), E (R_f 0.79), F (R_f 0.65). Amino acid analysis: Tyr 1.00, Gly 0.97, Ser 0.80, Phe 1.01, Leu 1.02.

6. Tyr-α-Aba-Gly-Phe-Leu - From Boc-Tyr(OBzl)-α-Aba-Gly-Phe-Leu-resin in 65% yield. TLC in solvent systems B (R_f 0.63), E (R_f 0.79), F (R_f 0.69). Amino acid analysis: Tyr 1.01, Gly 1.00, Phe 0.99, Leu 1.00.

7. Tyr-Gly-Phe-Leu - From Boc-Tyr-Gly-Phe-Leu-resin in 75% yield. TLC in solvent systems B (R_f 0.75), E (R_f 0.71), F (R_f 0.55). Amino acid analysis: Tyr 0.99, Gly 1.01, Phe 1.01, Leu 0.99.

8. Tyr-Ser-Gly-Phe-Leu - From Boc-Tyr(OBzl)-Ser(OBzl)-Gly-Phe-Leu-resin in 81% yield. TLC in solvent systems B (R_f 0.54), E (R_f 0.70), F (R_f 0.51). Amino acid analysis: Tyr 1.01, Ser 0.84, Gly 1.03, Phe 1.02, Leu 1.00.

Bioassay Methods. Melanocyte stimulating (MSH-like) activity was determined by the *in vitro frog skin assay using the photoreflectance method (20)*. Adenylate cyclase assays were performed using neuroblastoma x glioma cell homogenates as described by Sharma et al. (21).

RESULTS AND DISCUSSION

The solid phase synthesis of [Leu^5]enkephalin and its derivatives was readily accomplished and the peptides were easily cleaved from the resin with liquid HF (-COOH terminal), by trans-esterification (-COOMe terminal), or with ammonia (-$CONH_2$ terminal). Highly purified products (>98%) were obtained with a single or two gel filtration purifications in 60-80% yields.

Each peptide was tested for its ability to inhibit the morphine sensitive adenylate cyclase of neuroblastoma x glioma hybrid, NG108-15, cells. The results of these assays are expressed in Table I as concentration of peptide required for 50% of the maximal inhibition (Ki). [Leu^5]enkephalin was found to have a Ki of 25 nM, in good agreement with the results (40 nM) obtained with an independently synthesized sample of this peptide in earlier studies (22). Each of the analogues of [Leu^5]enkephalin prepared was of lower potency, although many were as effective or more effective than morphine.

The activity of [Leu^5]enkephalin is highly sensitive to changes at position 2. All substitutions for glycine studied including L-Ala (4), α-aminobutyric acid (6), deletion (7)[3] and L-serine (8) reduced the activity of [Leu^5]enkephalin. These results suggest that the conformation of the peptide required to fit the receptor is one which cannot be easily accomodated by an L-amino acid residue in position 2.

[3] The activities we report for compound 7 are consistent with a previous study (8) *in a different assay system.*

TABLE I

50% INHIBITORY CONCENTRATIONS OF PEPTIDES ON ADENYLATE CYCLASE ACTIVITY OF NEUROBLASTOMA X GLIOMA HYBRID CELLS

Peptide		Ki[a] (nM)	Activity[b] (%)
1	Tyr-Gly-Gly-Phe-Leu	25	100
2	Tyr-Gly-Gly-Phe-Leu-OMe	80	30
3	Tyr-Gly-Gly-Phe-Leu-NH_2	130	20
4	Tyr-Ala-Gly-Phe-Leu	1,500	2
5	Tyr-Gly-Ser-Phe-Leu	2,400	1
6	Tyr-αAba-Gly-Phe-Leu	5,000	0.5
7	Tyr-Gly-Phe-Leu	6,500	0.4
8	Tyr-Ser-Gly-Phe-Leu	30,000	0.1
	Tyr-Gly-Gly-Phe-Met	12[c]	250
	Morphine	1,500[c]	2

[a]Concentration required for 50% of maximal inhibition of the opiate sensitive adenylate cyclase activity in homogenates of neuroblastoma x glioma hybrid cells (21).

[b]Potency expressed relative to that of [Leu^5]enkephalin.

[c]Data taken from Klee and Nirenberg (22).

Interestingly, D-ala in position 2 of [Met^5]enkephalin has been reported to be a highly active analogue (3, 11). Substitution of glycine in position 3 seems much less deleterious to function than substitution in position 2 (compare compounds 5 and 8).

A free carboxylate group, while not critical to the acitivity of the peptides is nonetheless of some importance since both the ester 2 and the amide 3 are less potent than the free carboxylate compound in our assay. The ester 2 is about 3-fold less potent than [Leu^5]enkephalin, while the amide 3 is about 5-fold less active. The latter compound was reported to be about 3-fold weaker than [Leu^5]-enkephalin in a different assay system (3). These results indicate that the proposed interaction of the $-NH_3^+$ and $-COO^-$ terminal groups in enkephalin (15), considered a possible consequence of a β-turn structure, cannot be of essential importance in the biological activities of these compounds.

From the standpoint of the empirical rules of Chou and Fasman (23, 24) and the studies of Scheraga and co-workers (25, 26), the probability of an α-helix structure for enkephalin is small. Except for leucine, all the residues in [Leu^5]-enkephalin are very poor α-helical supporting residues. We find that substitution of the very poor α-helical supporting residue, glycine, by the very good α-helical supporting residues, alanine or α-aminobutyric acid, in position 2 of [Leu^5]enkephalin results in the less active enkephalin derivatives 4 and 6, respectively (Table 1). Based on these results and the NMR data (15-17), an α-helix structure for the enkephalins seems very unlikely.

As previously suggested for [Met^5]enkephalin, a 1→4 turn (β-turn) structure is a reasonable one for [Leu^5]enkephalin based on the Chou-Fasman rules (23, 24), and the work of Lewis et al. (25). Since removal of the glycine residue from [Leu^5]enkephalin as in 7 diminishes the probability of a β-bend, a significant decrease in Ki for this compound, could be considered consistent with a β turn structure. However in spite of a high probability for a serine residue to be located in a β-turn, neither [Ser^2,Leu^5]enkephalin, 8, nor [Ser^3,Leu^5]enkephalin, 5, show good activity. This might argue against a β turn structure for the receptor conformation of enkephalin, although we cannot rule out steric interference of the side chain with binding. In the latter regard, the large decrease in activity of the series 1>4>6>8 is of particular interest. It should be noted that when L-serine is located at the 2-position of enkephalin (compound 8), its hydroxyl group can be

in a very similar structural position to that of the aliphatic hydroxyl group of morphine and related opiates, and yet 8 has greatly reduced activity. In summary, the results in Table I, while not conclusive, provide little support for either an α-helical or β-turn structure as the major conformational state of receptor bound leucine enkephalins.

We have also tested the melanocyte stimulating (MSH-like) activity for these compounds using the frog skin bioassay systems and the photoreflectance method of Shizume et al. (20) to measure the skin darkening response to the compounds. Under conditions where α-MSH gives a detectible response at 10^{-11} molar, we get no activity for [Leu^5]enkephalin, 1, [Ala^2,Leu^5]enkephalin, 4, and [Ser^2,Leu^5]enkephalin, 8, in the concentration range 2×10^{-8} to 2×10^{-4} molar. Our results thus fail to support an MSH-like activity for the enkephalins as recently reported (12).

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REFERENCES

1. Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., and Morris, H. R. (1975), Nature, 258, 577-579.
2. Simantov, R. and Snyder, S. H., (1976), Life Sci. 18, 781-788.
3. Chang, J.-K., Fong, B. T. W., Pert, A. and Pert, C. B., (1976), Life Sci., 18, 1473-1481.
4. Bradbury, A. F., Smyth, D. G., Snell, C. R., Birdsall, N. J. M. and Hulme, E. C., (1976), Nature 260, 793-795.
5. Buscher, H. B., Hill, R. C., Romer, D., Cardinaux, F., Closse, A., Hauser, D. and Pless, J., (1976), Nature 261, 423-425.
6. Bradley, P. B., Briggs, I., Gayton, R. J. and Lambert, L. A., (1976), Nature 261, 425-426.
7. Gent, J. P. and Wolstencroft, J. H., (1976), Nature 261, 426-427.
8. Terenius, L., Wahlstrom, A., Lindeberg, G., Karlsson, S. and Ragnarsson, U., (1976), Biochem. Biophys. Res. Commun. 71, 175-179.
9. Simantov, R. and Snyder, S. H., (1976), Proc. Nat. Acad. Sci. U. S. A., 73, 2515-2519.
10. Minneman, K. P. and Iverson, L. L. (1976), Nature 262, 313-314.
11. Coy, D. H., Kastin, A. J., Schally, A. V., Morin, O., Caron, N. G., Labrie, F. Walker, J. M., Fertel, R., Berutson, G. G., and Sandmer, C. A., (1976), Bioche Biophys. Res. Commun., 73, 632-638.

12. Medzihradszky, M. and Medzihradszky-Schweiger, H. (1976), FEBS Letters 67, 45-47.

13. Goldstein, A., Goldstein, J. S. and Cox, B. M. (1975), Life Sciences, 17, 1643-1654.

14. Bradbury, A. F., Smyth, D. G. and Snell, C. R. (1976), Nature, 260, 165-166.

15. Roques, B. P., Garbay-Jaurequiberry, C., Oberlin, R., Anteunis, M. and Lala, A. K., (1976), Nature 262, 778-779.

16. Jones, C. R., Gibbons, W. A. and Garsky, V., (1976), Nature, 262, 779-782.

17. Bleich, H. E., Cutnell, J. D., Day, A. R., Freer, R. J., Glasel, J. A. and McKelvy, J. F., (1976), Proc. Nat. Acad. Sci. U. S. A., 73, 2589-2593; Bleich, H. E., Day, A. R., Freer, R. J., and Glasel, J. A., (1977), manuscript submitte

18. Merrifield, R. B., (1963), J. Amer. Chem. Soc., 85, 2149-2153.

19. Upson, D. A. and Hruby, V. J. (1976), J. Org. Chem. 41, 1353-1358.

20. Shizume, K., Lerner, A. B. and Fitzpatrick, T. B. (1954), Endocrinology 54, 553-560.

21. Sharma, S. K., Nirenberg, M., and Klee, W. A., (1975), Proc. Nat. Acad. Sci. U. S. A., 72, 590-594.

22. Klee, W. A., and Nirenberg, M., (1976), Nature, 263, 609-612.

23. Chou, P. Y. and Fasman, G. B., (1974), Biochemistry, 13, 211-222.

24. Chou, P. Y. and Fasman, G. B., (1974), Biochemistry, 13, 222-245.

25. Lewis, P. N., Momany, F. A. and Scheraga, H. A., (1971), Proc. Nat. Acad. Sci. U. S. A., 68, 2293-2297.

26. Tanaka, S., and Scheraga, H. A., (1976), Macromolecules, 9, 142-182.