Proc. Natl. Acad. Sci. USA
Vol. 77, No. 6, pp. 3211–3214, June 1980
Biochemistry

β-Endorphin omission analogs: Dissociation of immunoreactivity from other biological activities

(peptide synthesis/radioimmunoassay/analgesia/ileal opiate activity/opiate receptor-binding activity)

CHOH HAO LI*, DONALD YAMASHIRO*, LIANG-FU TSENG†, WEN-CHANG CHANG*, AND PASCUAL FERRARA*

*Hormone Research Laboratory, University of California, San Francisco, California 94143; and †Department of Pharmacology, The Medical College of Wisconsin, Milwaukee, Wisconsin 53233

Contributed by Choh Hao Li, March 5, 1980

ABSTRACT **An analog of human β-endorphin with omission of four residues at positions 11, 14, 20, and 22 has been synthesized. This analog and other synthetic analogs with deletion of a single amino acid at position 2, 5, 6, 10, 11, 12, 13, 15, or 22 have been assayed for analgesic potency, ileal opiate activity, opiate receptor-binding activity, and immunoreactivity. Results show that deletion of a single amino acid of the β-endorphin molecule outside of the enkephalin segment to give des-Gln^{11}-, des-Thr^{12}-, des-Pro^{13}-, des-Leu^{14}-, des-Val^{15}-, des-Asn^{20}-, or des-Ile^{22}-β-endorphin markedly reduced or abolished the immunoreactivity yet gave substantial retention of opiate potencies. Deletion of a single amino acid of β-endorphin within the enkephalin segment (des-Gly^{2}- or des-Met^{5}-β-endorphin) did not markedly affect the immunoactivity; however, the opiate activities were abolished or markedly reduced. The data indicate a clear dissociation of immunoactivity from analgesic, ileal-opiate, and opiate receptor-binding activities.**

β-Endorphin (β-EP) (ref. 1; see Fig. 1) is a naturally occurring opioid peptide with potent opiate analgesic activity after intracerebral (2) or intravenous injections (3, 4). Studies on structure–activity relationships indicate that the entire β-EP molecule is necessary for full analgesic potency (5). In addition, omission of a single amino acid residue at position 14 or 20 abolishes immunoreactivity yet gives retention of opiate potency (6). We present herein biological activities of synthetic analogs with deletion of a single amino acid at position 2, 5, 6, 10, 11, 12, 13, 15, or 22 as well as a synthetic analog with omission of four residues at positions 11, 14, 20, and 22. The analogs were assayed for analgesic activity by the tail-flick test, ileal opiate activity by the guinea pig ileum method, opiate receptor-binding activity by displacement of [^{3}H]-β-EP binding to membrane of rat brain, and immunoreactivity by radioimmunoassay. Results show a clear dissociation of immunoreactivity from other biological activities.

MATERIALS AND METHODS

Synthesis of single-deletion analogs of β_c-EP has been described (6, 7). Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP was synthesized by the solid-phase method (8). It was performed with Boc(Bzl)Glu brominated polymer (0.34 mmol/g) (4) on a Beckman model 990 peptide synthesizer. A fully automated symmetrical anhydride program (5) was used except for the Asn residue, which was incorporated by procedures described for the synthesis of β_h-EP (4). The following amino acid residues in the β_h-EP sequence were omitted in the synthesis: Gln^{11}, Leu^{14}, Asn^{20}, and Ile^{22}. From 295 mg (100 μmol) starting resin there was obtained, after removal of the last Boc group, 768 mg of protected peptide resin corresponding to des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP. Cleavage and deprotection in HF (9), gel filtration on Sephadex G-10 (0.5 M acetic acid), and chromatography on carboxymethylcellulose were performed as described (4). From additivity rules for the hydrophobicities of amino acid residues in β-EP (7) it could be predicted that partition chromatography on Sephadex G-50 in the same solvent system used for β_h-EP (4) would give an R_F of 0.27 based on the reported R_F of 0.40 for β_h-EP. The experimental value of R_F was found to be 0.26. The overall yield of des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP based on 50 μmol of starting resin was 39.0 mg (26%). The product (50-μg samples) was homogeneous on thin-layer chromatography on silica gel in 1-butanol/pyridine/acetic acid/H_2O (5:5:1:4, vol/vol), R_F 0.40 (ninhydrin and chlorine–tolidine detection), and in paper electrophoresis on Whatman 3 MM at pH 3.7 (R_F 0.58 relative to Lys) and pH 6.7 (R_F 0.45 relative to Lys) at 400 V (5 hr, ninhydrin detection). Amino acid analysis of a 24-hr HCl hydrolysate gave (theoretical values in parentheses): Lys, 4.91 (5); Asp, 1.06 (1); Thr, 3.12 (3); Ser, 1.92 (2); Glu, 2.10 (2); Pro, 0.96 (1); Gly, 2.97 (3); Ala, 2.12 (2); Val, 1.02 (1); Met, 0.98 (1); Ile, 1.00 (1); Leu, 1.07 (1); Tyr, 1.96 (2); Phe, 1.96 (2). Amino acid analysis of an enzymic digest (trypsin and chymotrypsin followed by leucine aminopeptidase) gave: Lys, 4.90 (5); Thr + Ser + Asn, 6.30 (6); Glu, 1.96 (2); Pro, 0.91 (1); Gly, 2.84 (3); Ala, 2.14 (2); Val, 1.12 (1); Met, 0.98 (1); Ile, 1.06 (1); Leu, 1.10 (1); Tyr, 1.90 (2); Phe, 1.84 (2).

```
                    5                   10
H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-

                   15                  20
Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-

                   25                          31
Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
```

FIG. 1. Amino acid sequence of β_h-EP (β_c-EP: His-27, Gln-31). Residues 1–5 correspond to [Met]enkephalin.

Opiate activities were assessed both *in vitro* and *in vivo*. The ileal opiate activity *in vitro* was measured by the inhibition of electrically stimulated contraction of guinea pig ileum preparation (10), and the opiate receptor-binding assay was performed according to the procedure recently described (11, 12), using [^{3}H-Tyr^{27}]-β_h-EP (13) as the primary ligand and synthetic

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: β-EP, β-endorphin (subscripts h and c indicate β-EP from human and camel pituitaries); IC_{50}, 50% inhibitory concentration.

Table 1. Ileal opiate activities of omission analogs of β-EP

Synthetic peptide	IC_{50},* nM	Relative potency
β_c-EP	91	100
Des-Gly^2-β_c-EP	9100	1
Des-Leu^{14}-β_c-EP	65	140
β_c-EP	58	100
Des-Thr^6-β_c-EP	60	97
Des-Ser^{10}-β_c-EP	49	118
Des-Thr^{12}-β_c-EP	50	116
β_c-EP	132	100
Des-Met^5-β_c-EP	2000	7
Des-Val^{15}-β_c-EP	107	123
Des-Ile^{22}-β_c-EP	67	197
β_h-EP	22	100
β_c-EP	22	100
Des-Gln^{11}-β_c-EP	22	100
Des-Pro^{13}-β_c-EP	20	110
Des-Asn^{20}-β_c-EP	20	110
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	16	138

* Guinea pig ileum assay; IC_{50} is the concentration that gives 50% inhibition of contraction.

β_c-EP (14) or β_h-EP (4) as standard competing ligand. The analgesic activity *in vivo* was assessed in mice by the tail-flick method (15) as described (5). Radioimmunoassay was carried out by the procedure described (16, 17).

RESULTS

The opiate activities *in vitro* of various omission analogs as assayed by the guinea pig ileum preparation are summarized in Table 1. Deletion of Gly^2 or Met^5 in the [Met]enkephalin segment of β-EP causes a marked decrease of opiate potency. On the other hand, deletion of a single amino acid residue outside the [Met]enkephalin segment does not alter the opiate potency and in some cases even enhances it. For example, des-Ile^{22}- and des-Leu^{14}-β_c-EP are 1.4 and 1.97 times as potent as the intact peptide, respectively. Omission of four residues in positions 11, 14, 20, and 22 increases the potency to 138% compared with β_h-EP.

Table 2. Analgesic potencies of omission analogs of β-EP

Synthetic peptide	AD_{50}*, nmol/mouse	Relative potency
β_c-EP	0.026 (0.020–0.032)	100
Des-Gly^2-β_c-EP	>25	<0.1
Des-Gln^{11}-β_c-EP	0.033 (0.021–0.048)	79
Des-Pro^{13}-β_c-EP	0.113 (0.089–0.149)	23
Des-Asn^{20}-β_c-EP	0.057 (0.042–0.075)	46
β_c-EP	0.043 (0.035–0.075)	100
Des-Met^5-β_c-EP	0.219 (0.099–0.487)	20
Des-Thr^6-β_c-EP	0.059 (0.048–0.077)	73
Des-Ser^{10}-β_c-EP	0.047 (0.027–0.092)	92
Des-Thr^{12}-β_c-EP	0.045 (0.024–0.095)	96
Des-Leu^{14}-β_c-EP	0.057 (0.033–0.093)	75
Des-Val^{15}-β_c-EP	0.179 (0.131–0.241)	24
Des-Ile^{22}-β_c-EP	0.075 (0.045–0.117)	57
β_h-EP	0.064 (0.026–0.17)	100
Des-Gln^{11},Leu^{14},Asn^{20}, Ile^{22}-β_h-EP	0.99 (0.45–2.19)	7

* Median antinociceptive dose (95% confidence limit).

Table 3. Opiate receptor-binding activities of omission analogs of β-EP

Synthetic peptide	IC_{50}, pM	Relative potency
β_c-EP	250	100
Des-Gly^2-β_c-EP	50,000	0.5
Des-Met^5-β_c-EP	12,000	2
Des-Thr^6-β_c-EP	550	45
Des-Ser^{10}-β_c-EP	280	90
Des-Gln^{11}-β_c-EP	210	120
Des-Thr^{12}-β_c-EP	270	93
Des-Pro^{13}-β_c-EP	860	29
Des-Leu^{14}-β_c-EP	430	58
Des-Val^{15}-β_c-EP	390	64
Des-Asn^{20}-β_c-EP	530	47
Des-Ile^{22}-β_c-EP	180	139
β_h-EP	560	100
β_c-EP	250	224*
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	610	92*, 41†

* Relative to β_h-EP.
† Relative to β_c-EP.

Table 2 presents the analgesic potencies *in vivo* of the synthetic analogs. Des-Gly^2-β_c-EP is less than 0.1% as potent as β_c-EP, and des-Met^5-β_c-EP exhibits only 20% potency relative to that of β_c-EP. The majority of analogs with deletion of a single amino acid residue outside the [Met]enkephalin segment retain substantial analgesic potency. However, deletion of four residues (positions 11, 14, 20, and 22) drops potency to 7% of that of the intact molecule.

As in the ileal opiate activity assay, the deletion of a single amino acid residue at position 2 or 5 markedly reduced the potency in the opiate receptor assay using membranes of rat brain. Deletion of a single amino acid residue outside the enkephalin segment, however, did not markedly alter the opiate receptor-binding potency (Table 3). Even deletion of four residues simultaneously had no drastic effects.

Table 4 summarizes the immunoreactivity of omission analogs by the β_h-EP radioimmunoassay system. Deletion of a single amino acid residue at position 11, 12, 13, 14, 15, or 20 abolished or markedly reduced the abilities of these peptides

Table 4. Immunoactivity of omission analogs of β-EP

Synthetic peptide	IC_{50},* pM	Relative activity
β_c-EP	72	100
Des-Gln^{11}-β_c-EP	800	9
Des-Pro^{13}-β_c-EP	1,029	7
Des-Leu^{14}-β_c-EP	>10,000	<1
Des-Val^{15}-β_c-EP	>10,000	<1
Des-Asn^{20}-β_c-EP	>10,000	<1
Des-Ile^{22}-β_c-EP	248	29
β_c-EP	56	100
Des-Gly^2-β_c-EP	68	82
β_h-EP	51	100
β_c-EP	51	100
Des-Met^5-β_c-EP	67	76
Des-Thr^6-β_c-EP	48	106
Des-Ser^{10}-β_c-EP	70	73
Des-Thr^{12}-β_c-EP	1,300	3
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	>10,000	<1

* Radioimmunoassay.

to bind to the antibodies of β-EP. On the other hand, omission of Gly^2 or Met^5 in the [Met]enkephalin segment of β_c-EP as well as Thr^6 or Ser^{10} resulted in retention of high immunoreactive potency.

DISCUSSION

Previous studies indicated the importance of the Tyr^1, Phe^4, and Met^5 residues for the production of opiate analgesic activity (18–20). In this study, we found that deletion of Gly^2 or Met^5 in the [Met]enkephalin segment of β-EP drastically lowers opiate analgesic, ileal opiate, and receptor-binding potency, whereas nearly full immunoreactivity is retained. Of these two residues, Gly^2 appears to be more important for the production of opiate activities. Omission of a single residue outside of this segment does not cause considerable loss of biological activities, but immunoreactivity is markedly affected. Omission of a single amino acid at position 14, 15, or 20 abolishes immunoreactivity yet retains significant amounts of other biological activities. This indicates that the active sites in the β-EP molecule for binding to the β-EP antibodies resides in positions 11 to 22. Thus we have discovered an instance in which deletion of a single amino acid residue in a biologically active peptide abolishes immunoreactivity.

Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP has virtually no immunoreactivity and exhibits somewhat higher ileal opiate and significant receptor-binding activity in comparison to β_h-EP. Analgesic potency of this analog is only 7% when compared with the activity for the intact molecule. In an earlier report (16), a lack of correlation between immunoreactivity and opiate activity as assayed by the guinea pig ileum preparation has been noted.

When the relative ileal opiate activities for des-Gln^{11}-, des-Leu^{14}-, des-Asn^{20}-, and des-Ile^{22}-β_c-EP are averaged, a value of 137 is obtained. It is interesting that the observed opiate activity of des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP is 138. On the other hand, the similarly calculated values for the other activities of des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP (receptor-binding activity, 124; analgesic potency, 64, and immunoreactivity, 10) diverge increasingly from the experimental data in the order given. These results illustrate the insensitivity of the ileal assay to such structural alterations, while the other assays show sensitivity in the order immunoreactivity > analgesic activity > receptor-binding activity.

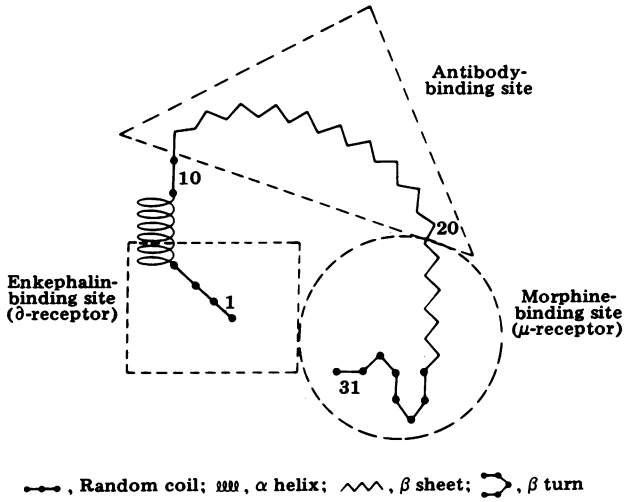

FIG. 2. Proposed binding sites in the primary structure of β-EP. Predicted secondary structure of β_h-EP was taken from ref. 23.

The data, summarized in Table 5, clearly show the dissociation of immunoreactivity from analgesic, ileal opiate, and receptor-binding activities. Moreover, there is a fair correlation between analgesic potency and receptor-binding activity if des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP is excluded. This omission analog possesses significant receptor-binding activity and low analgesic potency. The lack of correlation between opiate receptor-binding activity and analgesic potency has recently been observed with synthetic analogs with extension at the COOH terminus (21). These data emphasize again the importance of not relying on a single assay procedure for the characterization of biologically active peptides.

There are at least two receptors for opioid peptides in the brain (22): the μ receptors for morphine and the ∂ receptors for the enkephalins. The data presented herein, together with the recent findings that β_c-EP-(6–31) and β_c-EP-(20–31) segments inhibit morphine-induced analgesia (unpublished), suggest the presence of three binding sites in the β-EP molecule as shown in Fig. 2. The first site resides in the [Met]enkephalin segment [enkephalin-binding site ("∂-receptor")] and the second consists

Table 5. Relative biological activities of omission analogs of β-EP

Synthetic peptide	Analgesic potency	Opiate activity	Receptor-binding activity	Immunoreactivity
β_c-EP	100	100	100	100
Des-Gly^2-β_c-EP	<0.01	1	0.5	82
Des-Met^5-β_c-EP	20	7	2	76
Des-Thr^6-β_c-EP	73	97	45	106
Des-Ser^{10}-β_c-EP	92	118	90	73
Des-Gln^{11}-β_c-EP	79	100	120	9
Des-Thr^{12}-β_c-EP	96	116	93	3
Des-Pro^{13}-β_c-EP	23	110	29	7
Des-Leu^{14}-β_c-EP	75	140	58	<1
Des-Val^{15}-β_c-EP	24	123	64	<1
Des-Asn^{20}-β_c-EP	46	110	47	<1
Des-Ile^{22}-β_c-EP	57	197	139	29
β_h-EP	100	100	100	100
β_c-EP	100	100	224*	100
Des-Gln^{11},Leu^{14},Asn^{20},Ile^{22}-β_h-EP	7	138	92*, 41†	<1

* Relative to β_h-EP.
† Relative to β_c-EP.

of the COOH-terminal segment [β-EP-(21–31)] [morphine-binding site ("μ-receptor")]. The middle segment [β-EP-(11–20)] is the antibody-binding site. Studies on the *in vivo* and *in vitro* biological profiles of synthetic β-EP analogs may possibly clarify the role of these binding sites.

We thank K. Hoey and N. Shine for technical assistance. This work was supported in part by grants from the National Institute of Mental Health (MH-30245 to C.H.L.), National Institute of General Medical Sciences (GM-2907 to C.H.L.), National Institute of Drug Abuse (DA-02352 to L.-F.T.), and the Hormone Research Foundation.

1. Li, C. H. & Chung, D. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1145–1148.
2. Loh, H. H., Tseng, L.-F., Wei, E. & Li, C. H. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2895–2898.
3. Tseng, L.-F., Loh, H. H. & Li, C. H. (1976) *Nature (London)* **263,** 239–240.
4. Li, C. H., Yamashiro, D., Tseng, L.-F. & Loh, H. H. (1977) *J. Med. Chem.* **20,** 325–328.
5. Li, C. H., Tseng, L.-F. & Yamashiro, D. (1978) *Biochem. Biophys. Res. Commun.* **85,** 795–800.
6. Li, C. H., Chang, W.-C., Yamashiro, D. & Tseng, L.-F. (1979) *Biochem. Biophys. Res. Commun.* **87,** 693–697.
7. Yamashiro, D. (1979) *Int. J. Pept. Protein Res.* **13,** 5–11.
8. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85,** 2149–2154.
9. Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M. & Sugihara, H. (1967) *Bull. Chem. Soc. Jpn.* **40,** 2164–2167.
10. Kosterlitz, H. W., Lydon, R. T. & Watt, A. F. (1970) *Brit. J. Pharmacol.* **39,** 398–413.
11. Ferrara, P., Houghten, R. & Li, C. H. (1979) *Biochem. Biophys. Res. Commun.* **89,** 786–792.
12. Ferrara, P. & Li, C. H. (1980) *Int. J. Pept. Protein Res.*, in press.
13. Houghten, R. A. & Li, C. H. (1978) *Int. J. Pept. Protein Res.* **12,** 325–326.
14. Li, C. H., Lemaire, S., Yamashiro, D. & Doneen, B. A. (1976) *Biochem. Biophys. Res. Commun.* **71,** 19–25.
15. D'Amour, F. E. & Smith, D. L. (1941) *J. Pharmacol. Exp. Ther.* **72,** 74–79.
16. Li, C. H., Rao, A. J., Doneen, B. A. & Yamashiro, D. (1977) *Biochem. Biophys. Res. Commun.* **75,** 576–580.
17. Chang, W.-C., Yeung, H. W. & Li, C. H. (1979) *Int. J. Pept. Protein Res.* **13,** 278–281.
18. Yamashiro, D., Tseng L.-F., Doneen, B. A., Loh, H. H. & Li, C. H. (1977) *Int. J. Pept. Protein Res.* **10,** 159–166.
19. Yamashiro, D., Li, C. H., Tseng, L.-F. & Loh, H. H. (1978) *Int. J. Pept. Protein Res.* **11,** 251–257.
20. Blake, J., Tseng, L.-F., Chang, W.-C. & Li, C. H. (1978) *Int. J. Pept. Protein Res.* **11,** 323–328.
21. Li, C. H., Tseng, L.-F., Ferrara, P. & Yamashiro, D. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2303–2304.
22. Kosterlitz, H. W. (1978) in *Endorphins '78*, eds. Gráf, L., Palkovito, M. & Ronai, A. J. (Academiai Kiado, Budapest, Hungary), p. 205.
23. Jibson, M. D. & Li, C. H. (1979) *Int. J. Pept. Protein Res.* **14,** 113–122.