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SYNTHETIC *N*-DIMETHYL β-ENDORPHIN, A STABILIZED OPIOID PEPTIDE*

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Abstract—A procedure for the dimethylation of the amino groups of human β-endorphin by reductive methylation is described. A single product with the theoretical maximum degree of methylation was produced and the consequences of dimethylation on proteolytic attack are reported. The derivative was shown to be resistant to tryptic digestion and to attack by leucine aminopeptidase. The nonmethylated β-endorphin was rapidly degraded by incubation with pituitary homogenate; under these conditions methylated β-endorphin was degraded at a slower rate. In the presence of bacitracin, the methylated peptide was essentially resistant to degradation by the pituitary homogenate. The methylated peptide may be expected to have a longer *in vivo* half-life.

Peptides with an N-terminal sequence corresponding to methionine-enkephalin (Tyr–Gly–Gly–Phe–Met) account for a series of endogenous opioid peptides related to β-lipotropin [1]. The fragment of this peptide known as β-endorphin (β-lipotropin residues 61–91) is the most potent opiate in this series of peptides [2–8]. It is the only one of these peptides producing deep, long-lasting analgesia, and the only naturally occurring opioid peptide that is active following systemic administration [9]. The pentapeptide enkephalins have little or no analgesic activity when administered *in vivo* [8, 10–13], despite their opioid activity on various *in vitro* systems. This is widely assumed to be due to the rapid biodegradation of the enkephalins by aminopeptidase activity [14–17] and cleavage at the Gly–Phe bond [18–20].

With both enkephalins and β-endorphin, the loss of the N-terminal tyrosine leads to a complete loss of opiate receptor activity. The greater *in vivo* potency of administered β-endorphin may, in part, be linked to the slower susceptibility to aminopeptidase attack characteristic of the full 31 amino acid sequence [17, 21]. Anticipating that further stability might lead to further *in vivo* potency, we have investigated the degradation of β-endorphin after dimethylation of the amino groups. It is established with some proteins that dimethylation of the ε-amino group of lysine renders the derivative essentially resistant to tryptic digestion [22]; methylation at the N-terminal α-amino group may afford protection against aminopeptidase attack. The β-endorphin sequence contains five lysine residues but no arginine, so that dimethylation of the ε-amino group of lysine may confer complete protection against tryptic digestion. At physiological pH the methylated amino groups will have a positive charge close to that of the unmodified amino group. Protection against proteolytic attack may possibly be conferred with little change in pharmacological potency, as suggested by a recent report which demonstrates that the methylated peptide retains the receptor binding affinities of the native compound [23]. In the present study, we demonstrate that the methylated endorphin shows a high degree of protection against proteolytic digestion, both with purified proteolytic enzymes and with crude pituitary homogenates.

MATERIALS AND METHODS

Materials. [^{14}C]Formaldehyde (52–56 mCi/mmole) and carrier-free Na^{125}I (17 Ci/mg) was purchased from the New England Nuclear, Corp., Boston MA. Sodium cyanoborohydride was purchased from the Aldrich Chemical Co., Milwaukee, WI, soybean and ovomucoid trypsin inhibitor, bacitracin, and leucine aminopeptidase from the Sigma Chemical Co., St. Louis, MO, trypsin (TPCK-treated)‡ from the Worthington Biochemical Corp., Freehold, NJ, and β-lipotropin (61–69) from Peninsula Laboratories, San Carlos, CA. Rats were Sprague–Dawley males, weighing 220–240 g, from Simonsen Laboratories, Inc., Gilroy, CA. Thin-layer plates (silica, 0.25 mm) were purchased from Brinkmann Instruments (Westburg, NY) and the reverse phase C_{18} Ultrasphere–ODS column was purchased from Altex, Palo Alto, CA.

Synthetic human β-endorphin was a gift of Dr. C. H. Li, University of California, San Francisco.

Iodination of β-endorphin. The labeling of β-endorphin by iodination of tyrosine residues was by the chloramine T method, with all reagents in 0.1 M potassium phosphate buffer, pH 7.4. To 25 μl of Na^{125}I (0.32 nmole) was added 10 μl of β-endorphin (containing 10 μg) and 10 μl of chloramine T (2 mg/ml). After 10 sec at room temperature, 20 μl of $Na_2S_2O_5$ (2 mg/ml) was added, and after 10 min the peptide was separated from unreacted iodine on a Sephadex G-10 column eluted with 50% acetic

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‡ TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone.

acid. The pooled β-endorphin fractions had a specific activity of 100–135 Ci/mmole.

Reductive methylation of β-endorphin. Reductive methylation was carried out in a fume hood by an adaptation of the procedure of Jentoft and Dearborn [24]. To 30 μl of β-endorphin (1 μg/μl) in H_2O was added 50 μl of 200 mM Hepes* buffer (pH 7.5), 30 μl of formaldehyde (2 mM final concentration), and 10 μl of sodium cyanoborohydride (200 mM). Incubation was for 2 hr at 37°. The reaction was stopped by the addition of 10 μl of KBH_4 (2.7 mg/ml in 10 mM KOH) and, after 5 min, 20 μl of 1 N HCl. The mixture was layered onto a Sephadex G-10 column, equilibrated, and eluted as described for the iodination procedure.

Either ^{14}C or ^{125}I was introduced into this procedure, as appropriate. The degree of methylation was first assessed by using [^{14}C]formaldehyde in the reaction. The specific activity of the product was calculated after liquid scintillation counting of the column effluent, and the efficiency of methylation was then assessed by comparing the resulting specific radioactivity with the theoretical maximum (which is about 0.7 Ci/mmole with 100 per cent methylation). The method produced a specific radioactivity of 0.63 to 0.74 Ci/mmole. This indicates that close to 100 per cent dimethylation occurred. The resulting labeled peptide was analyzed by reverse phase high-pressure liquid chromatography (h.p.l.c.), sodium dodecylsulphate–polyacrylamide gels, gel exclusion chromatography, and carboxymethyl cellulose ion exchange chromatography. In all four cases, only one labeled compound was detected. In the case of the acrylamide gels and the gel exclusion chromatography, this single labeled species was shown to comigrate exactly with Coomassie blue staining of authentic β-endorphin or with iodinated β-endorphin.

Having established the efficiency of the procedure, the reaction was performed using nonradioactive formaldehyde but with about 5×10^6 cpm of iodinated β-endorphin added to the reaction mixture, generating methylated iodinated β-endorphin ([^{125}I]-Me-β-endorphin).

Tissue preparation and incubation conditions. Entire pituitaries (weighing about 10 mg) were dissected and immediately homogenized by sonication in 10 vol. of 25 mM potassium phosphate buffer, pH 7.4. The incubation mixture was then made up with 10 μl of homogenate, 20 μl of 0.5 M Tris buffer (pH 8.3), 10 μl of [^{125}I]-β-endorphin or [^{125}I]-Me-β-endorphin (approximately 100×10^3 cpm per tube giving a final molarity of 6 μM) and 10 μl of water or inhibitor dissolved in water, as appropriate. In some instances the homogenate was replaced by trypsin or leucine aminopeptidase (20 μg in 80 μl incubation volume, preincubated for 2 hr with manganese chloride). Bacitracin (1 mM final concentration) or ovomucoid trypsin inhibitor (1 μg/μl) was added, as appropriate. Incubation was for 10 min at 37°, and was stopped by the addition of 50 μl of 50% acetic acid. After a 5-min centrifugation at 12,000 *g*, the supernatant fraction was removed to a fresh tube and usually 1.5 to 3 μl was applied to a thin-layer plate, which was run in butanol–ethyl acetate–acetic acid–H_2O (1:1:1:1) or butanol–H_2O–methanol (4:3:1) for a distance of 16.5 to 17 cm. The silica gel was then scraped in 5 mm segments and counted in a Packard gamma counter.

Reverse phase h.p.l.c. Products of incubation were applied in 25% acetic acid to a C_{18} Ultrasphere–ODS column (0.66 × 25 cm, 5 μm) and eluted at 1.5 ml/min with an acetonitrile gradient in 50 mM ammonium acetate (pH 4.0). The gradient was 5–20 per cent (5 min), 20–50 per cent (25 min), 50–80 per cent (20 min). Standards were detected by absorbance at 277 nm, and 1-min fractions were collected for gamma counting.

RESULTS

Figure 1B shows the thin-layer chromatography of iodinated β-endorphin after a 24-hr incubation with trypsin (trypsin:peptide ratio 1:400). The degradation of the β-endorphin under these conditions can be estimated by comparison with Fig. 1A,

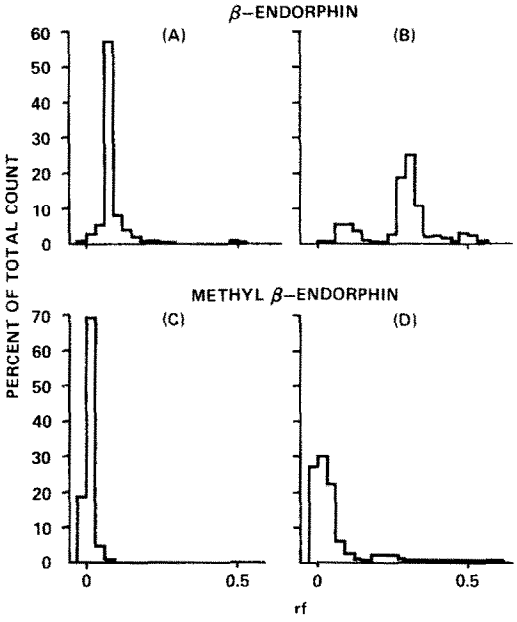

Fig. 1. Digestion of β-endorphin and methyl β-endorphin by trypsin. Incubations were as described in Materials and Methods. Iodinated β-endorphin (6 μM) was the substrate for the top row (A, B) and iodinated methyl β-endorphin (6 μM) for the bottom row (C, D). All incubation times were for 16 hr with trypsin (enzyme:peptide ratio 1:400) in B and D, and no enzyme in A and C. Incubations were followed by thin-layer chromatography on silica using a solvent system of butanol–H_2O–methanol (4:3:1). The position of the origin (O) and the 0.5 R_f mark are shown. None of the radioactivity had an R_f of over 0.6 and so this part of the thin layer is not represented. Following chromatography the distribution of radioactivity was determined by scraping the gel in 0.5 mm segments and counting in a gamma counter. The radioactivity in each segment is presented here as a percentage of the total radioactivity counted in that lane.

* Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

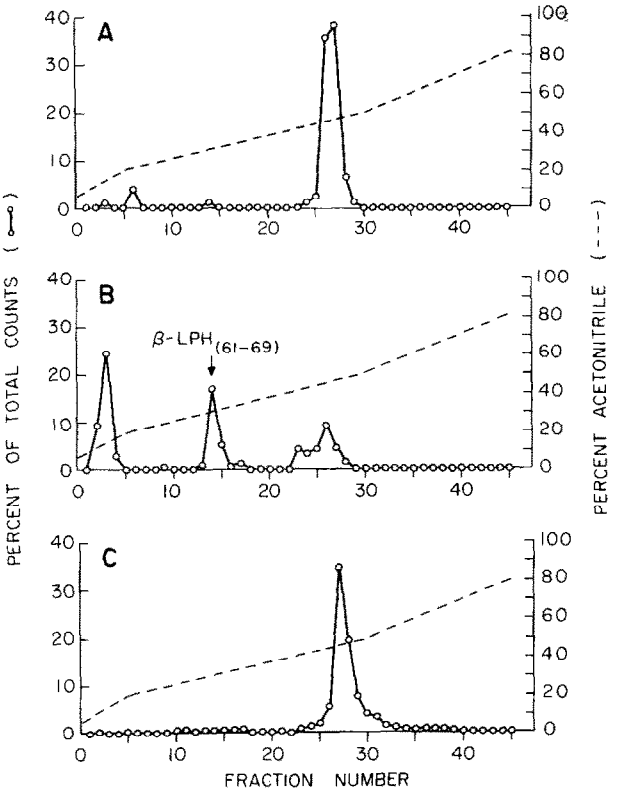

Fig. 2. Digestion of β-endorphin and methyl β-endorphin by trypsin. Iodinated β-endorphin (6 μM) was the substrate for A and B, and iodinated methyl β-endorphin (6 μM) for C. Incubation times were for 4 hr with trypsin (enzyme:peptide ratio 1:200) in B and C, and no enzyme in A. Incubations were followed by reverse phase h.p.l.c. on a C_{18} Ultrasphere–ODS column. One-minute fractions were taken (flow rate 1.5 ml/min) and the radioactivity in each was expressed as a percentage of the total (solid line). The acetonitrile gradient is shown (broken line).

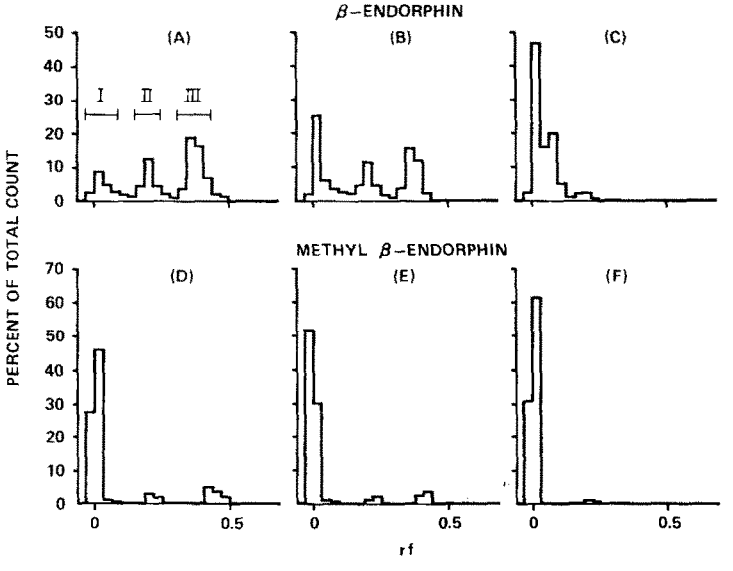

Fig. 3. Digestion of β-endorphin and methyl β-endorphin by pituitary homogenate. The experiments were performed and displayed as described in the legend to Fig. 1 and Materials and Methods, with the following conditions. The solvent system was butanol–H_2O–methanol (4:3:1). All incubations were for 5 min with pituitary homogenate, using iodinated β-endorphin as the substrate in the top row (A, B, C), and iodinated methyl β-endorphin in the bottom row (D, E, F). A and D had no inhibitors added, B and E had ovomucoid trypsin inhibitor (1 μg/μl), and C and F had bacitracin (1 mM).

when trypsin was omitted from an identical incubation. The consequences of the same incubation conditions on the methylated derivative can be seen by comparison of Fig. 1D (with trypsin) with Fig. 1C (no trypsin). It can be seen that the methylated peptide was largely resistant to the tryptic digestion that breaks down the nonmethylated peptide. In both cases the chromatographic pattern of the peptide incubated in the absence of the enzyme was the same as that applied directly to the thin layer with no prior incubation. Using this system the nonmethylated peptide moved just past the origin whereas the methylated peptide remained on the origin. Our experiments using reduction with dithiothreitol suggest that this difference was not due to differential oxidation of the methionine to the sulphoxide form. The tryptic digestion of β-endorphin and methyl β-endorphin was compared further using a reverse phase h.p.l.c. separation system. The results of one such experiment with a 4-hr incubation (trypsin:peptide ratio 1:200) are shown in Fig. 2. These conditions resulted in digestion of the majority of β-endorphin, whereas methyl β-endorphin showed essentially complete resistance to tryptic digestion. The pK values of dimethylamino groups are about 0.5 units below that of the primary amino groups so that with this reverse phase separation (pH 4) no charge difference between β-endorphin and methyl β-endorphin should be apparent. The alkylation of amino groups will, however, result in a small increase in hydrophobicity, explaining the slightly longer retention time (about 0.5 min) seen with the methylated peptide in this system. Using the reverse phase system (Fig. 2B), we identified β-LPH$_{61-69}$ as a major labeled product of tryptic digestion of β-endorphin; the other major peak that eluted shortly after the void volume was probably the tetrapeptide Asn–Ala–Tyr–Lys close to the C-terminal of the human β-endorphin, and the small amount of radioactivity that eluted immediately before β-endorphin was probably the βh-LPH$_{61-88}$ peptide (also known as C′). The formation of these products of tryptic digestion was prevented by prior methylation (Fig. 2C).

Similar experiments were performed with leucine aminopeptidase in place of trypsin, using incubations of 15, 30 and 60 min at room temperature. Under these conditions, in which the nonmethylated β-endorphin is not stable, the methylated form was found to be resistant to breakdown. The thin-layer analysis produced results similar to those shown for tryptic digestion.

Degradation of β-endorphin during a 5-min incubation with pituitary homogenate can be estimated by comparing Fig. 3A with Fig. 1A. Under these conditions the rapid degradation of β-endorphin can be observed; this was not seen if the pituitary homogenate was inactivated previously by heating to 100°. The degree of proteolysis of the methylated form, Fig. 3D, can be seen to be considerably less than the breakdown of the nonmethylated peptide. The β-endorphin remaining at the origin of the thin layer was about 23 per cent of the total after incubation with pituitary extract; the equivalent figure for the methylated form was over 75 per cent of the peptide remaining at the origin. A similar result for a 10-min incubation can be seen in Table 1. The stability of the methylated form was verified (data not shown) using a second thin-layer solvent system for separation (butanol–ethyl acetate–H_2O–methanol at a 1:1:1:1 ratio) and using reverse phase h.p.l.c.

The digestion by the crude pituitary extracts was rapid compared to those reported in the literature using other tissue preparations. To provide some preliminary indications as to the nature of the protection against pituitary homogenate proteolysis that is afforded to the peptide by methylation, we investigated the effect of adding bacitracin (an aminopeptidase inhibitor) and ovomucoid trypsin inhibitor to the incubation. The results are shown in histogram form in Fig. 3 for a 5-min incubation and in percentage figures in Table 1 for a 10-min incubation. The percentage figures in Table 1 refer to the percentage of total counts in each of three regions of the thin layer designated in Fig. 3A. The first region, close to the origin, included intact β-endorphin, while the other two (II and III) included two groups of degradation products. At both 5 and

Table 1. β-Endorphin and methyl β-endorphin breakdown by pituitary homogenate during a10-min incubation*

Substrate peptide	Treatment	Per cent of total counts on thin-layer region		
		I	II	III
β-Endorphin				
	Control	25.5	17.6	41.5
	Trypsin inhibitor	42.9	14.7	29.0
	Bacitracin	81.0	7.2	0
Methyl β-endorphin				
	Control	64.0	8.6	17.7
	Trypsin inhibitor	72.6	5.8	13.0
	Bacitracin	98.0	2.0	0

* Iodinated β-endorphin or methyl β-endorphin was incubated (at 6 μM) with pituitary homogenate for 10 min and then separated on a silica thin layer using butanol–H_2O–methanol (4:3:1). The Roman numerals I, II and III refer to the regions of the thin layer designated in Fig. 3A, using the same solvent system. The counts in each region are expressed as a percentage of the total counts on each lane. The ovomucoid trypsin inhibitor was at 1 μg/μl and the bacitracin at 1 mM.

10 min the nonmethylated peptide was protected to a small degree by ovomucoid trypsin inhibitor and to a much greater degree by bacitracin. With the methylated peptide the same was true of the smaller amount of degradation which occurred. The trypsin inhibitor had a small effect, whereas the methylated peptide was essentially resistant to proteolysis by pituitary homogenate in the presence of bacitracin during a 10-min incubation (Table 1).

DISCUSSION

These results show that dimethylation of amino groups conferred protection on β-endorphin molecules against both tryptic and aminopeptidase attack, when the enzymes were supplied in pure form from a commercial source. In the presence of a crude pituitary homogenate, the methylated derivative was found to have a considerably increased protection against those proteolytic enzymes that rapidly degraded the nonmethylated form of the opioid peptide. Although degradation of β-endorphin in the presence of brain preparation has been studied [25, 26], this rapid degradation in a crude pituitary homogenate has not been investigated previously. We demonstrated that the majority of this proteolysis was inhibited by bacitracin, and a smaller degree of protection was afforded by ovomucoid trypsin inhibitor (Fig. 3). This suggests that aminopeptidases are principally involved in the rapid degradation, with trypsin-like endopeptidases effecting a small degree of breakdown with 5- and 10-min incubations. It therefore seems most likely that the high degree of protection against this pituitary breakdown conferred by methylation is a result of protection against both aminopeptidase and endopeptidase attack. The breakdown of methyl β-endorphin that did occur in the presence of pituitary homogenate appeared mainly due to aminopeptidase attack, because this compound appeared completely stable in the presence of bacitracin.

The high degree of protection against tryptic-like endopeptidase digestion provided by methylation is a result of multiple lysines and a complete absence of arginine in the peptide. The protection afforded against aminopeptidase attack in pituitary homogenates may be ascribed to direct protection by α-amino methylation and, possibly, indirectly by a reduction in the rate of chain shortening by endopeptidase attack as a result of lysine methylation, since it has been suggested that the shorter sequences are better substrates for aminopeptidase digestion [21].

The methylation of an amino group led to only a minor change in the pK of that group and in many cases there was little change in the activity of proteins following methylation. In the case of opioid peptides, the methylated derivative may be expected to retain opiate activity. This has been observed in the case of α-N-methylation of enkephalin, when the derivative has an affinity for receptor similar to that of the parent compound [27] and, in the case of β-endorphin, when receptor binding is retained after dimethylation of amino groups [23]. β-Endorphin is the most active, *in vivo*, of the opioid peptides, possibly due to its greater resistance to proteolysis [21]. The demonstration in this paper that methylated β-endorphin was more stable than the nonmethylated peptide suggests that it may have had a longer half-life following administration and, therefore, greater *in vivo* potency. That methylated β-endorphin is stabilized against proteolytic attack suggests that this compound may prove useful in both *in vitro* and *in vivo* studies involving the use of opioid peptides.

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