ANALYTICAL BIOCHEMISTRY 141, 1–9 (1984)

Quantitation of the Endopeptidase Activity Generating γ-Endorphin from β-Endorphin in Rat Brain Synaptic Membranes by a Radiometric Assay

JOS L. M. LEBOUILLE,[1] J. PETER H. BURBACH, AND E. RONALD DE KLOET

Rudolf Magnus Institute for Pharmacology, Medical Faculty, University of Utrecht, Vondellaan 6, 3521 GD Utrecht, The Netherlands

Received October 11, 1983

γ-Endorphin is a naturally occurring biologically active peptide that is produced by an endopeptidase activity cleaving its precursor β-endorphin. This enzyme was termed γ-endorphin generating enzyme (γ-EGE). In order to quantitate γ-EGE activity by means of a simple and sensitive assay two synthetic peptides derived from the sequence surrounding the γ-EGE cleavage site in β-endorphin were tested as substrates. One of these peptides Ac-Val–Thr–Leu–Phe–Lys-$NHCH_3$ fulfilled all criteria for a suitable γ-EGE substrate. The peptide was exclusively cleaved at the correct bond for γ-EGE upon incubation with brain synaptic membranes, and this cleavage was inhibited by the naturally occurring substrate β-endorphin. The peptide was insensitive to cleavage by exopeptidases and cathepsin D. Addition of a ^{14}C-labeled methyl group at the lysine residue of this peptide by reductive methylation did not alter its properties as a substrate for γ-EGE activity. The use of the ^{14}C-labeled peptide allowed sensitive quantitation of its radioactive products after simple separation by hydrophobic chromatography on minicolumns containing polystyrene beads. γ-EGE activity increased linearly with a protein concentration and incubation time. This assay can be used for reliable quantitation of γ-EGE activity and permits investigations on the regulation of γ-endorphin production.

KEY WORDS: neuropeptides; brain endopeptidase; cathepsin D; hydrophobic absorption chromatography; radiometric assay; reductive methylation.

γ-Endorphin is a biologically active peptide of the brain and the pituitary gland that has distinct effects in various behavioral and pharmacological test situations (1–5). Biosynthesis of γ-endorphin involves enzymatic cleavage of its precursor β-endorphin. Previously we have identified in a synaptic membrane preparation an endopeptidase activity able to convert β-endorphin directly into γ-endorphin by cleaving the Leu^{17}–Phe^{18} bond of β-endorphin (6–9). We term this enzyme activity γ-endorphin-generating enzyme (γ-EGE).[2] Investigations on the production of γ-endorphin at the level of the γ-EGE were initiated by developing a reliable assay for this enzyme activity. Our approach was to test a synthetic peptide derived from the sequence surrounding the γ-EGE cleavage site in β-endorphin as substrate in a radiometric assay (see Fig. 1). Such a peptide should meet a number of criteria. First, incubation with synaptic membranes should result in initial cleavage of the Leu–Phe bond of the peptide. Second, cleavage should be inhibited by the naturally occurring substrate β-endorphin in order to prove competition for the same enzyme. Third, the peptide should be protected against exopeptidases. And fourth, the peptide should provide specificity in measuring γ-EGE by distinguishing between γ-EGE and cathepsin D, an enzyme which also can cleave the Leu–Phe bond of β-endorphin (10,11) and which occurs as a lysosomal contamination in subcellular fractions of brain tissue (12). In addition, for high sensitivity of the assay the peptide should carry a radioactive label, while

[1] To whom correspondence should be sent.

[2] Abbreviation used: γ-EGE, γ-endorphin-generating enzyme.

0003-2697/84 $3.00

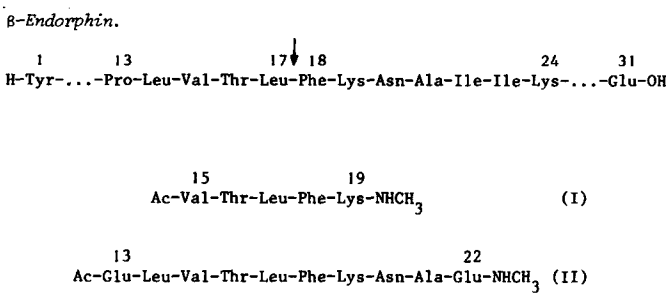

FIG. 1. Amino acid sequence of human β-endorphin-(1–31) and analogs of the midportion of β-endorphin [Ac-Val[15],Lys[19]-$NHCH_3$]β-endorphin-(15–19) (I) and [Ac-Glu[13],Glu[22]-$NHCH_3$]β-endorphin-(13–22) (II). The cleavage site in β-endorphin for the conversion into γ-endorphin is indicated by the arrow.

quantitative resolution of products of γ-EGE action should be rapid and reproducible.

This article presents an assay for γ-EGE that fulfills all the above-mentioned requirements. The preparation of a ^{14}C-labeled and C- and N-terminal-protected peptide is described and the development of a chromatographic system for the rapid separation of γ-EGE products from the intact peptide is described. The assay permits reliable quantitation of γ-EGE activity in synaptic membranes of rat brain.

MATERIALS AND METHODS

Chemicals. Carboxypeptidase A (EC 3.4.2.1) from bovine pancreas and cathepsin D (EC 3.4.23.5) from bovine spleen were purchased from Sigma Chemical Company (St. Louis). Aminopeptidase M (EC 3.4.11.2) from porcine kidney microsomes was a commercial preparation from Boehringer (Mannheim, W. Germany). [^{14}C]Formaldehyde with a specific activity of 53 μCi/μmol was purchased from New England Nuclear (Boston), and anhydrous sodium cyanoborohydride was obtained from Aldrich-Europe (Beerse, Belgium). Amberlite XAD-2 came from BDH Chemicals (Poole, England). Pippet tips were purchased from Labsystems Oy (Helsinki, Finland). The peptides [Ac-Val[15],Lys[19]-$NHCH_3$]β-endorphin-(15–19) (peptide I) and [Ac-Glu[13],-Glu[22]-$NHCH_3$]β-endorphin-(13–22) (peptide II) were synthesized and generously donated by Dr. H. M. Greven and Dr. J. W. van Nispen from Organon International B.V. (Oss, The Netherlands).

Preparation of the [$^{14}CH_3$]-peptides. Radioactive labeling by reductive methylation of the peptides I and II (into peptide [$^{14}CH_3$]-I and peptide [$^{14}CH_3$]-II) was performed by a procedure based on earlier reports (13–16). Optimal utilization of [^{14}C]formaldehyde was achieved under the following conditions: reaction of 0.8 nmol peptide, 0.8 nmol [^{14}C]formaldehyde, and 16 nmol sodium cyanoborohydride was carried out in a volume of 800 μl 25 mM sodium phosphate buffer, pH 7.5, at 60°C for 4 h. The reaction was stopped in ice. Separation between methylated and nonmethylated peptides was performed by a single reverse-phase HPLC step. Nonradioactive methylation was performed under conditions that resulted in complete methylation of peptides I and II, by increasing the formaldehyde concentration to 16 nmol.

Incubations. Incubations of the peptides I and II with carboxypeptidase A, aminopeptidase M, and cathepsin D were carried out with 100 nmol peptide and 500 pmol peptidase in a volume of 800 μl (100 mM) sodium acetate buffer, pH 4.0 (cathepsin D), or sodium phosphate buffer, pH 8.0 (aminopeptidase M and carboxypeptidase A) at 37°C. After digestion for different time intervals the mixture was applied to HPLC and the eluate was monitored by uv detection.

To analyze the products of peptide [CH_3]-I and [CH_3]-II generated by proteolytic activity

in synaptic membranes 100 nmol methylated peptide was incubated with synaptic membranes (1.75 mg protein/ml) in a volume of 1.0 ml (100 mM) sodium phosphate buffer, pH 7.4, at 37°C for 3 h. The reaction was stopped by boiling.

Assay procedure for γ-EGE. In subsequent order we designed incubations for the peptidase assay. As an enzyme source synaptic membranes prepared as described previously were used. Briefly rat forebrains were homogenized and debris was removed as a pellet by a 1000*g* centrifugation step. The pellet of the subsequent 10,000*g* centrifugation step was lysed with water. The supernatant of the 10,000*g* centrifugation step following this lysis contained light synaptic membranes (9). This fraction was washed and pelleted. Incubations were performed with a final protein concentration of 1.0 mg/ml in a total volume of 50 μl (25 mM) sodium phosphate buffer, pH 7.4, at 37°C. The incubations were started by adding 10,000 dpm peptide [$^{14}CH_3$]-I (final concentration 0.86 μM) and performed in duplo. After 30 min the incubations were stopped by addition of 50 μl of 2 M acetic acid and subsequent boiling for 10 min. After boiling, 400 μl (10 mM) ammonium acetate buffer, pH 4.15, or 10% ethanol in the same buffer was added to the samples. It was not found necessary to remove membranes before chromatography. Samples were sometimes stored at −20°C before analysis, which did not interfere with the results. Separation of peptide [$^{14}CH_3$]-I and its products was performed by hydrophobic chromatography on polystyrene beads (Amberlite XAD-2) based on an earlier report (17). Columns containing 400 mg polystyrene were dry-packed in 1000-μl pipet tips and equilibrated by subsequent portions of 2 ml of 100% ethanol, 2 ml of 50% ethanol in 10 mM ammonium acetate buffer, pH 4.15, and 10% ethanol in the same buffer. Columns were kept for multiple use without change of chromatographic characteristics. The last equilibration step could also be performed with 100% of the buffer. Samples of 500 μl were loaded on the columns and products were eluted first by 4 portions of 500 μl of 10% ethanol containing 10 mM ammonium acetate buffer, pH 4.15. Subsequently the intact peptide was eluted with 2 ml 90% ethanol. The two eluates were collected separately in counting vials. Enzymatic activity was determined on basis of radioactivity in both vials by liquid scintillation counting with an efficiency of 89%.

High-pressure liquid chromatography. The HPLC equipment was from Waters Associates as previously described (9). HPLC was performed on a reverse-phase μBondapak C_{18} column (0.39 × 30 cm: Waters Associates) eluted with gradients of 10 mM ammonium acetate, pH 4.15 (solvent A) and 1.5% acetic acid in methanol (solvent B). Chromatography of digestion products was performed with a gradient of increasing solvent B with 1% per min. The gradient was started with the injection of the digest. For the separation of the methylated and nonmethylated peptides 100 mM ammonium acetate, pH 6.0, with 0.13% heptafluorobutyric acid (solvent C) and with 0.1% heptafluorobutyric acid and 1.5% acetic acid in methanol (solvent D) was applied. The elution was started at 45% solvent D with an increase of 1% solvent D per min starting with the injection. Ultraviolet was monitored at 1.0 AUFS. Effective change of the solvents appeared after 5 min in the uv spectrum.

Amino acid analysis. Amino acid analysis was based on HPLC of amino acids modified by precolumn derivatization with a *o*-phthaldialdehyde-2-mercaptoethanol reagent as previously described (18). Separation of amino acid derivates was done on a Cp–Spher C_8 column (250 × 4.6 mm i.d.) (Chrompack, Middelburg, The Netherlands). Hydrolysis was performed in 6 M hydrochloric acid containing 0.5% thioglycolic acid in evacuated sealed glass tubes at 110°C for 16 h. Hydrolysates were dried and dissolved in 1% sodium dodecyl sulfate, according to Jones *et al.* (19). Elution was performed by a stepwise gradient of 2% tetrahydrofan (20) in 100 mM sodium citrate buffer, pH 6.0, (solvent E) and methanol (solvent F) at a flow rate of 1.5 ml/min running

from 10 to 65% solvent F over 32 min, interrupted by an isocratic plateau between 12 and 20 min.

RESULTS

Preparation of ^{14}C-Labeled Peptide

The reaction of peptide I (Fig. 1) with [^{14}C]formaldehyde yielded two labeled products as detected by HPLC (Fig. 2). Component 3 which contained 27% of the total amount of radioactivity was completely separated from component 2 and the nonlabeled peptide I (component 1). HPLC analysis of component 3 using an isocratic run of 50% solvent A and 50% solvent B (see Materials and Methods) showed one peak with 98.3% of the total radioactivity. The residual 1.7% eluted in the void volume. Amino acid analysis of component 3 demonstrated that the lysine residue was derivatized. The specific activity of the lysine residue in this peptide was calculated to be 118 μCi/μmol, showing that the lysine contained two methyl groups. This double labeling of the lysine residue has been described previously (21). We assume that component 2 is the mono-methylated derivate. All assays with the radioactive peptide I were performed with the double-methylated peptide (component 3) which we indicate as peptide [$^{14}CH_3$]-I. Optimal utilization of ^{14}C radioactivity (27%) in the reductive methylation reaction was achieved by carrying out the reaction with equimolar concentrations of [^{14}C]formaldehyde and peptide and excess of sodium cyanoborohydride at elevated temperature (60°C). Increasing the incubation time above 4 h did not improve the incorporation of radioactivity.

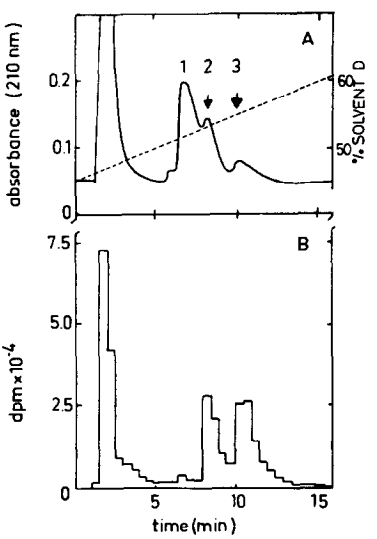

FIG. 2. Purification of peptide [$^{14}CH_3$]-I by reverse-phase HPLC. Profiles of uv absorbance (210 nm) (1.0 AUFS) (A) and radioactivity (B) of products obtained after reductive methylation of peptide I with [^{14}C]-formaldehyde and sodium cyanoborohydride. Component 1 coelutes with peptide I. Components 2 and 3 are radioactively labeled (B). Component 3 represents the double-methylated derivative of peptide I. It had a specific activity of 118 μCi/μmol as analyzed by quantitative amino acid analysis and radioactivity measurement. Component 2 is assumed to be the mono-methylated derivative. Solvent D: 0.1% heptafluorobutyric acid and 1.5% acetic acid in methanol.

Cleavage of the Peptides by Purified Enzymes and Brain Tissue Fractions

Both peptides I and II were very poorly converted by aminopeptidase M and carboxypeptidase A (Figs. 3A and B), under conditions that rapidly converted equimolar amounts of γ-endorphin ($t_{1/2}$ = 2 min). This indicated that the peptides I and II were effectively protected against exopeptidase action. The peptides differed in degradation rate upon incubation with the endopeptidase cathepsin D. Peptide II was converted for 93% by cathepsin D within 60 min, while peptide I was not significantly converted in this period. Even after 16 h of incubation 82% of peptide I remained intact (Fig. 3C). Similar results were obtained when peptide [$^{14}CH_3$]-I and peptide [$^{14}CH_3$]-II were incubated with a cathepsin D-containing fraction from rat brain tissue. Incubation of peptide [$^{14}CH_3$]-I with a 10,000g supernatant of a Whittaker P_2 lysate (9) (1.3 mg protein/ml), pH 4.0, for 30 min resulted only in a slight decrease in the amount of the peptide [$^{14}CH_3$]-I while peptide [$^{14}CH_3$]-II was converted for 53% (Fig. 4A). Using a brain

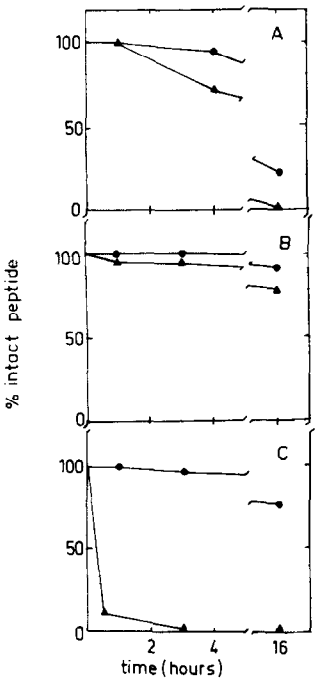

FIG. 3. Conversion of peptide I (●) and peptide II (▲) by carboxypeptidase A (A), aminopeptidase M (B), and cathepsin D (C). Incubations were performed in a substrate to enzyme ratio of 200:1 (molar). Intact peptide was quantitated by the uv absorbance (210 nm) (0.4 AUFS) in the HPLC elution profile.

fraction known to contain γ-EGE activity, i.e., synaptic membranes incubating at pH 7.4 (6–9) the two peptides [$^{14}CH_3$]-I and [$^{14}CH_3$]-II did not differ significantly in conversion rate (Fig. 4B). This indicates that both peptides were similarly converted by the proteolytic activity. The results show that peptide [$^{14}CH_3$]-II is cleaved by cathepsin D and γ-EGE, while peptide [$^{14}CH_3$]-I is exclusively cleaved by the γ-EGE activity. This renders peptide [$^{14}CH_3$]-I as a specific substrate for measurement of γ-EGE activity in tissues without interference of cathepsin D activity.

Initial Cleavage Site of the Substrate Peptides

To determine the initial site of cleavage in the peptides by proteolytic activity in synaptic membranes, peptides [CH_3]-I and [CH_3]-II were incubated with the membrane preparation at pH 7.4 and products were isolated by HPLC. Figure 5 shows the HPLC fractionation of a digest of peptide [CH_3]-I. Fractions A and B, the only detectable products, were collected and analyzed. Fraction C coeluted with intact peptide [CH_3]-I. The amino acid composition of fraction B was $Val_{0.95}$, $Thr_{1.10}$, $Leu_{1.15}$, and the fraction A contained only Phe. These results showed that peptide [$^{14}CH_3$]-I was initially cleaved at the Leu–Phe bond.

The peptide [CH_3]-II showed multiple cleavage sites. Four products were isolated from the digest. Two main fragments contained Glu, Leu, Val, Thr, Leu and Phe, Lys, Asn, Ala, Glu, respectively, indicating that this peptide was cleaved at the Leu–Phe bond. But the two other fragments containing Glu, Leu, Val, Thr and Leu, Phe, Lys, Asn, Ala, Glu, respectively, demonstrated that initial cleavage also occurred at the Thr–Leu bond. From these results and the insensitivity to cathepsin D it was concluded that peptide [$^{14}CH_3$]-I was an outstanding substrate for our assay.

Quantitation of γ-EGE Products

H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ is generated from peptide [$^{14}CH_3$]-I by γ-EGE activity. This H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ can be converted further into H-[$^{14}CH_3$]Lys-$NHCH_3$ by aminopeptidase activity present in synaptic mem-

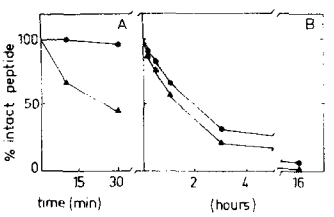

FIG. 4. Comparison between the susceptibilities of peptide [$^{14}CH_3$]-I (●) and peptide [$^{14}CH_3$]-II (▲) to cleavage by cathepsin D (A) and γ-EGE (B) in rat brain tissue. As a source for cathepsin D a 10,000*g* supernatant of the lysate of a Whittaker P_2 fraction was used at pH 4.0 (A). γ-EGE activity was contained in a synaptic membrane preparation incubated at pH 7.4 (B).

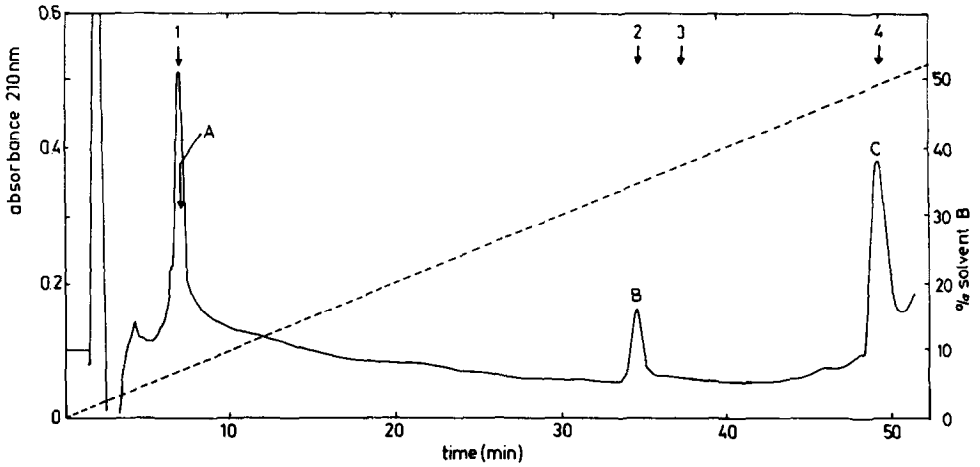

FIG. 5. HPLC of digestion products obtained by incubation of peptide [CH_3]-I with a synaptic membrane fraction at pH 7.4. The mobile phase was monitored by uv absorbance (210 nm). Fractions A, B, and C were collected for determinations of amino acid composition. The vertical numbered arrows indicate the elution position of (1) Phe, (2) Ac-Val-Thr-Leu, (3) Ac-Val-Thr-Leu-Phe, and (4) Ac-Val-Thr-Leu-Phe-Lys-$NHCH_3$. Solvent B: 1.5% acetic acid in methanol.

branes (6). Thus, H-[$^{14}CH_3$]Lys-$NHCH_3$ can occur as a secondary product of γ-EGE activity. Therefore, chromatography was designed to separate intact peptide [$^{14}CH_3$]-I from the two radioactive products H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ and H-[$^{14}CH_3$]Lys-$NHCH_3$. Separation of H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ from peptide [$^{14}CH_3$]-I was achieved by hydrophobic chromatography on Amberlite XAD-2 columns. After elution with 0% ethanol 47.1% of H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ eluted from the column, while no detectable amount of intact peptide did elute at this concentration. When 10% ethanol was used 98.3% of H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ was eluted while only 1% of the intact peptide was found in the 10% ethanol eluate. With 10% ethanol H-[$^{14}CH_3$]Lys-$NHCH_3$ was completely eluted from the column. Subsequent elution of the column with 90% ethanol eluted the intact peptide for 99%. Thus by stepwise elution by 10 and 90% ethanol the products of γ-EGE can be efficiently separated from nondigested substrate. Column efficiency was calculated to be approximately 95% on basis of recovered radioactivity in the eluates. In a typical experiment blanks were approximately 150–200 dpm.

Properties of γ-EGE

The dependency of the γ-EGE activity on protein concentration, incubation time, and pH was determined. γ-EGE activity increased in a linear proportion with protein concentration up to 4 mg/ml at an incubation time of 30 min (Fig. 6). Enzymatic activity was in linear relation with incubation time for the first 90 min at a 1.0-mg/ml protein concentration (Fig. 7). It was noted that the enzyme activity deviated from a linear relationship with protein concentration and incubation time when more than 50% of the peptide [$^{14}CH_3$]-I was converted. The conversion of peptide [$^{14}CH_3$]-I was optimal at pH 8.5 (Fig. 8) while no activity was shown below pH 5.5.

Inhibition of γ-EGE Action on Peptide [$^{14}CH_3$]-I by β-Endorphin

In an experiment that employed β-endorphin, the natural substrate for γ-EGE, to inhibit the conversion of peptide [$^{14}CH_3$]-I it was investigated whether peptide [$^{14}CH_3$]-I was recognized by the γ-EGE activity. The conversion of peptide [$^{14}CH_3$]-I could be inhibited up to 80% by increasing concentrations of β-endorphin (Fig. 8). The IC_{50} appeared to be

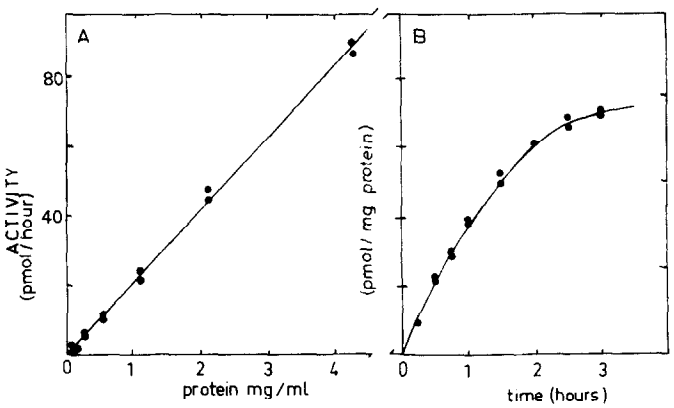

FIG. 6. (A) γ-EGE activity as function of membrane protein concentration. The incubation time was 30 min. Determinations were performed as described under Materials and Methods. (B) γ-EGE activity as function of incubation time. The protein concentration was 1.0 mg/ml. Determinations were performed as described under Materials and Methods.

about 30 μM with a concentration of peptide [$^{14}CH_3$]-I of 0.83 μM.

DISCUSSION

This paper describes a quantitative and specific assay for γ-EGE, which is an endopeptidase that converts *in vitro* β-endorphin to its biologically active fragment γ-endorphin. β-Endorphin itself could not be used as an appropriate substrate for the assay since endopeptidases also cleave β-endorphin at the Ala^{21}–Ile^{22} bond (9). As a consequence disappearance of β-endorphin could not solely reflect γ-EGE activity. Therefore a pentapeptide Ac-Val-Thr-Leu-Phe-Lys-NHCH$_3$ derived from the β-endorphin midportion was synthesized. This peptide contained the γ-EGE cleavage site and was protected against exopeptidases by derivatization of the N- and C-terminal amino acids. This peptide fulfilled a number of criteria. It was possible to discriminate between γ-EGE and cathepsin D activity. The peptide was cleaved at a single

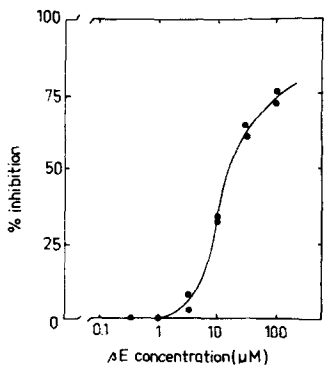

FIG. 7. Inhibition of γ-EGE activity in synaptic membranes as measured by using peptide [$^{14}CH_3$]-I (0.83 μM) as substrate by human β-endorphin-(1–31) with an IC_{50} of 30 μM. Determinations were performed as described under Materials and Methods.

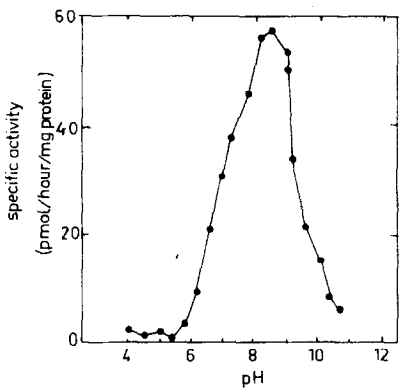

FIG. 8. γ-EGE activity as function of pH. Incubations were performed with 1.0 mg/ml membrane protein for 30 min. The buffers used were sodium phosphate/sodium citrate (pH 4.0–7.0), Tris/sodium hydroxide (pH 7.0–9.0), and sodium carbonate (pH 9.0–10.8). Determinations were performed as described under Materials and Methods.

internal bond and was resistant to exopeptidases. The peptide was labeled with ^{14}C and radioactive products arising after γ-EGE activity could simply be separated from the intact peptide.

A potential hazard in studying the γ-EGE activity is cathepsin D. This enzyme is present as a lysosomal contamination in subcellular fractions (12). Cathepsin D has previously been shown to cleave the same Leu–Phe bond in β-endorphin as γ-EGE (10,11) peptide I and its radioactively methylated derivative are not significantly converted by cathepsin D derived from bovine pancreas and rat brain. The absence of interference of cathepsin D in the assay for γ-EGE is also apparent from the absence of conversion of the substrate at pH 4.0 (Fig. 8) which is the pH optimum of cathepsin D. As another candidate peptide II (Ac-Glu–Leu–Val–Thr–Leu–Phe–Lys–Asn–Ala–Glu-$NHCH_3$) was synthesized. However, this peptide is rapidly converted by cathepsin D of bovine pancreas and rat brain. This indicates that the cleavage site Leu–Phe is recognized by cathepsin D in peptide II but not in peptide I. This phenomenon may be explained by observations of Barát *et al.* that a cathepsin D cleavage is often proceeded by the Leu–Val moiety (22). The Leu–Val moiety is modified in peptide I, which could explain why peptide I is insensitive to cathepsin D. Cathepsin D is inactive at the pH optimum of γ-EGE (8.5).

The intact peptide should initially be cleaved by γ-EGE only. Thus it should be insensitive to exopeptidases which are abundant in tissue fractions. Peptide I was not significantly degraded by exopeptidases. Some conversion was observed by carboxypeptidase A ($t_{1/2}$ = 5 h), however the rate is much lower than that of γ-endorphin which is not C-terminal-protected ($t_{1/2}$ = 2 min). Possibly it may even be that degradation by carboxypeptidase A is due to contamination of the enzyme preparation by some endopeptidase activity.

The assay for γ-EGE is based on the cleavage of the radioactive peptide substrate, followed by quantitation of radioactive products and remaining substrate. Thus it was essential to characterize carefully the site of initial cleavage in the peptide and to design a separation procedure that allowed separation between intact peptide and the mixture of radioactive products, i.e., H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ and H-[$^{14}CH_3$]Lys-$NHCH_3$.

The initial cleavage of peptide I by the membrane preparation was exclusively at the Leu–Phe bond since the tripeptide Ac-Val–Thr–Leu-OH was the only detectable fragment. The fragment Ac-Val–Thr–Leu–Phe could not be detected. It is concluded that the free Phe is derived from H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ by aminopeptidase activity present in synaptic membranes. Aminopeptidase activity in synaptic membranes has an optimum at pH 7.4 (22). Such secondary cleavage of initial products by aminopeptidases is also observed with β-endorphin (9). It was observed that after proteolysis of β-endorphin, β-endorphin-(18–31), the complementary fragment of γ-endorphin was rapidly degraded by aminopeptidase activity after cleavage of the Leu^{17}–Phe^{18} bond. This secondary cleavage does not interfere with the measurement of γ-EGE activity, since aminopeptidase cleavage can only occur after γ-EGE action. The products of aminopeptidase action H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ and H-[$^{14}CH_3$]Lys-$NHCH_3$ are quantitated together once separated from the intact peptide. For the separation of the products H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ and H-[$^{14}CH_3$]Lys-$NHCH_3$ from the intact peptide the procedure of Vogel *et al.* (17) was used. As the hydrophobic residue Phe was present in one of the two products a 0% ethanol elution was not efficient enough for complete elution (24). Using 10% ethanol a complete elution of H-Phe-[$^{14}CH_3$]Lys-$NHCH_3$ was achieved while the intact peptide remained on the column. The procedure is simple and rapid to perform, and turned out to be a reliable way to quantitate the enzyme activity.

The inhibition of peptide [$^{14}CH_3$]-I conversion by β-endorphin pointed out that the enzyme-cleaving peptide [$^{14}CH_3$]-I also rec-

ognized β-endorphin. This finding provides a strong argument that the enzyme activity as measured by our assay acted on both peptide [$^{14}CH_3$]-I and β-endorphin, thus being the γ-EGE activity as previously defined (7–9).

The relationships between enzyme activity and protein concentration, and incubation time were linear over a large range. In all assays with the peptide [$^{14}CH_3$]-I it was observed that conversion deviated from a linear relationship when the peptide was converted for more than 50%. It may be that the products generated by γ-EGE inhibit the reaction. Therefore, the assay is performed under conditions that not more than 50% of the peptide is converted. As conditions were routinely chosen a protein concentration of 1 mg/ml and an incubation time of 30 min.

The assay described in this paper was developed on basis of some known properties of the γ-EGE activity. It was known that the enzyme was present in synaptic membranes, that it was distinct from cathepsin D and that it showed activity at pH 7.4. By using the assay we could now establish that the optimal pH of the γ-EGE is pH 8.5. The assay thus opens the possibility to study properties and distribution of γ-EGE activity as well as its functional role in the metabolism of endorphins. Preliminary observations suggest that the enzyme activity can be influenced by endocrine manipulation.

ACKNOWLEDGMENTS

The authors thank Dr. H. M. Greven and Dr. J. W. van Nispen who synthesized and kindly donated the peptides used in this study. They also thank Miss E. Vergeer and Mrs. M. Markerink-van Ittersum for their technical assistance.

This study was supported by the foundation for Fundamental Medical Research (FUNGO), which is subsidized by the Netherlands organization for Advancement of Pure Research (ZWO) Project 13 49 26.

REFERENCES

1. De Wied, D., and Jolles, J. (1982) *Physiol. Rev.* **62,** 976–1059.
2. De Wied, D. (1978) *in* Characteristics and Function of Opioids (Van Ree, J. M., and Terenius, L., eds.), pp. 113–122, Elsevier/North-Holland Biomedical Press, Amsterdam.
3. De Wied, D., Kovacs, G. L., Bohus, B., Van Ree, J. M., and Greven, H. M. (1978) *Eur. J. Pharmacol.* **49,** 427–436.
4. Van Ree, J. M., Bohus, B., and De Wied, D. (1980) *in* Endogenous and Exogenous Opiate Agonists and Antagonists (Way, E. L., ed.), pp. 459–462, Pergamon, New York.
5. Burbach, J. P. H., and De Wied, D. (1980) *in* Enzymes and Neurotransmitters in Mental Disease: Based on a Symposium held at the Technion Faculty of Medicine, Haifa, Israel August 28–30, 1979 (Udsin, E., Sourkes, T. L., and Youdim, M. B. H., eds.), Wiley, New York.
6. Burbach, J. P. H., and De Kloet, E. R. (1979) *Peptides* **3,** 451–453.
7. Burbach, J. P. H., Loeber, J. G., Verl oef, J., Wiegant, V. M., De Kloet, E. R., and De Wied, D. (1980) *Nature (London)* **283,** 96–97.
8. Burbach, J. P. H., Loeber, J. G., Verhoef, J., and De Kloet, E. R. (1980) *Biochem. Biophys. Res. Commun.* **92,** 725–732.
9. Burbach, J. P. H., De Kloet, E. R., Schotman, P., and De Wied, D. (1981) *J. Biol. Chem.* **256,** 12,463–12,469.
10. Gráf, L., Kennessy, A., Patthy, A., Grynbaum, A., Marks, N., and Lathja, A. (1979) *Arch. Biochem. Biophys.* **193,** 101–109.
11. Benuck, M., Grynbaum, A., Cooper, T. B., and Marks, N. (1978) *Neurosci. Lett.* **10,** 3–9.
12. Tonnaer, J. A. D. M., Wiegant, V. M., and De Jong, W. (1982) *J. Neurochem.* **38,** 1356–1364.
13. Ascoli, M., and Puett, D. (1974) *Biochem. Biophys. Acta* **371,** 203–210.
14. Dottavio-Martin, D., and Ravel, J. M. (1978) *Anal. Biochem.* **87,** 562–565.
15. Jentoft, N., and Dearborn, D. G. (1979) *J. Biol. Chem.* **254,** 4359–4365.
16. Hammonds, R. G., Ling, N., and Puett, D. (1981) *Biochem. Biophys. Res. Commun.* **98,** 211–218.
17. Vogel, Z., and Altstein, M. (1977) *FEBS Lett.* **80,** 332–336.
18. Burbach, J. P. H., Prins, A., Lebouille, J. L. M., Verhoef, J., and Witter, A. (1982) *J. Chromatogr.* **237,** 339–343.
19. Jones, B. N., Pääbo, S., and Stein, S. (1981) *J. Liq. Chromatogr.* **4,** 565–586.
20. Hodgin, J. C. (1979) *J. Liq. Chromatogr.* **2,** 1047–1059.
21. Witter, A., Greven, H. M., and De Wied, D. (1975) *J. Pharmacol. Exp. Ther.* **193,** 853–860.
22. Barát, E., Patthy, A., and Gráf, L. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6120–6123.
23. Burbach, J. P. H., De Kloet, E. R., and De Wied, D. (1980) *Brain Res.* **202,** 401–414.
24. Niederwieser, A. (1971) *J. Chromatogr.* **54,** 215–223.