Hydrolysis of thymic humoral factor $\gamma 2$ by neutral endopeptidase (EC 3.4.24.11)

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A search for the natural substrates for neutral endopeptidase (NEP; EC 3.4.24.11) in the immune system led to investigation of the enzyme's action on thymic humoral factor $\gamma 2$ (THF). The ectoenzyme rapidly and efficiently hydrolyses the Lys⁶–Phe⁷ bond of the octapeptide. The site of cleavage was confirmed by h.p.l.c. analysis, amino acid analysis and sequence determination of the products. Phosphoramidon (3.6 μ M), a potent inhibitor of the enzyme, prevents this cleavage even during prolonged incubation. The high efficiency of hydrolysis of THF by NEP is similar to that reported for [Leu⁵]enkephalin, and the dipeptide Phe-Leu is the C-terminal product in the hydrolysis of both peptides. The presence of NEP, reportedly identified as the common acute lymphoblastic leukaemia antigen (CALLA), in bone-marrow cells and other cells of the immune system raises the possibility that it may play a role in modulating the activity of peptides such as THF.

INTRODUCTION

Neutral endopeptidase (NEP; EC 3.4.24.11) or enkephalinase is an ectoenzyme present on the surface of many cell types and is widely distributed in mammalian tissues, including the kidney and brain tissue, and in the immune system [1–3]. The amino acid sequence of human NEP is identical with that of the common acute lymphoblastic leukaemia antigen (CALLA, CD 10) [4,5] and is highly conserved in different species [6]. Catalytically, NEP shares basic elements of specificity and mechanistic properties with the well-characterized bacterial neutral proteinase thermolysin [7–9]. The enzyme is implicated in the metabolism and regulation of a variety of peptides, including the enkephalins, Substance P and atrial natriuretic factor [10]. In view of the presence of NEP in the immune system, we have undertaken the exploration of the putative substrates of the enzyme among the immunologically active peptides. Thymic humoral factor $\gamma 2$ (THF) is an immunoregulator peptide present in thymic extracts [11,12], recently identified as an octapeptide of the structure Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu [13]. THF augments T-cell functions such as the response to T-cell lectins and mixed lymphocyte reactions; it also increases interleukin-2 production by T-cells [13]. THF has been used as an immunomodulator in clinical conditions associated with immune impairment and dysregulation [14]. NEP rapidly hydrolyses the Lys⁶–Phe⁷ bond of the peptide with a high catalytic efficiency comparable with that reported for the enzyme acting on [Leu⁵]enkephalin [15], suggesting a common mechanism for the inactivation of these two peptides by NEP.

MATERIALS AND METHODS

Materials

Synthetic THF was prepared by the solid-phase procedure [13,16]. Phe-Leu was prepared as previously described [17]. Phosphoramidon was from Peptide Institute (Osaka, Japan).

NEP from bovine kidney was purified by Triton X-100 extraction followed by DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden), concanavalin A-Sepharose (Bio-Yeda, Rehovot, Israel), Q-Sepharose (Pharmacia) and hydroxyapatite (Bio-Rad Laboratories, Richmond, CA, U.S.A.) chromatographic procedures [18]. The molar concentration of the purified enzyme was estimated by protein determination [19], and a molecular mass of 90 kDa was deduced from SDS/PAGE [20]. NEP activity was assayed by a two-stage enzymic assay with the synthetic substrate 3-carboxypropanoylalanylalanyl-leucine 4-nitroanilide (Suc-Ala-Ala-Leu-NH-Np) supplemented with *Streptomyces griseus* aminopeptidase I [18,21].

Hydrolysis of THF by NEP

THF was incubated at 37 °C with 0.48 μ g of NEP/ml in 0.2 or 0.4 ml of 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl. At the times indicated 20–50 μ l samples were removed, heated in boiling water for 3 min, cooled rapidly and centrifuged briefly before being subjected to h.p.l.c.

For the kinetic studies the concentration of THF was varied from 15 μ M to 800 μ M and the concentration of NEP was 0.24 μ g/ml. Initial rates were calculated from the first 10 min of reaction. Zero-time points were taken immediately after the addition of enzyme and heat inactivation. Kinetic parameters were derived from a Lineweaver–Burk plot.

H.p.l.c. analysis of THF cleavage

H.p.l.c. analysis was carried out isocratically with a Waters h.p.l.c. system consisting of a model 510 pump, a model 441 Absorbance Detector and a model 730 Data Module, at 0.5 ml/min and 23 °C with detection at 214 nm. Separation of THF and hydrolysis products was carried out on a 5 μ M-particle-size LC-18 reverse-phase column (4.6 mm $\times$ 250 mm) (Supelco). Solvent was 50 mM-orthophosphoric acid / triethylamine (pH 3.1)/acetonitrile (7:3 or 13:7, v/v). Identification of peaks was facilitated by injecting Phe-Leu and THF before injection of the sample. The rate of THF hydrolysis was computed from the

Abbreviations used: NEP, neutral endopeptidase (EC 3.4.24.11); THF, thymic humoral factor $\gamma 2$; Suc-, 3-carboxypropanoyl-; -NH-Np, 4-nitroanilide.

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peaks of the Phe-Leu product, by establishing calibration curves of the peak areas with the use of various amounts of Phe-Leu.

Amino acid composition analysis and sequence determination

Freeze-dried samples of THF and its hydrolysis products, eluted from the h.p.l.c. system, were hydrolysed by HCl vapour at 110 °C for 24 h and vacuum-dried. The samples were analysed on a Dionex 4000 amino acid analyser. Amino acid sequence analysis was performed with a model 470A gas-phase micro-sequencer equipped with an on-line model 120A phenylthiohydantoin analyser (Applied Biosystems) as described previously [13].

Inhibition studies

The inhibition by THF of NEP-catalysed hydrolysis of the synthetic substrate Suc-Ala-Ala-Leu-NH-Np was assessed over a range of concentrations of 0.1–0.4 mM-substrate and 0–0.12 mM-THF. To 1.5 ml of substrate at the appropriate concentration at 37 °C in 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl and 10 mM-CaCl₂ was added 0.1 ml of THF in water. After removal of the zero-time sample (0.35 ml), 2 µl (0.05 µg) of NEP was added and incubation was immediately initiated. At 1, 6 and 11 min after the addition of NEP, 0.35 ml samples were removed, heated in boiling water for 3 min and cooled to room temperature. After centrifugation and preincubation for 5 min at 37 °C, 4 µl of *Streptomyces griseus* aminopeptidase I solution (1.6 mg/ml) was added, and the samples were mixed and then incubated for 10 min at 37 °C. Then 0.3 ml of each sample was transferred into microplate wells (Nunc-Immuno Plate Maxi-Sorp F96) and absorbance was measured at 405 nm. Initial rates were derived from the increase in absorbance between 1 and 6 min of reaction.

RESULTS

Hydrolysis of THF by NEP

Incubation of THF (0.96 mM) with NEP (5.3 nM) results in the formation of two distinct products (Fig. 1a). THF itself (peak II) and the C-terminal dipeptide of THF, Phe-Leu (peak III), are clearly identified by their elution volumes compared with standard samples of these peptides (Figs. 1b and 1c respectively). Peak I is presumably the N-terminal hexapeptide Leu-Glu-Asp-Gly-Pro-Lys (see below). Thus NEP cleaves THF uniquely at the Lys⁶-Phe⁷ bond, thereby conforming with the basic specificity element of the enzyme, which is known to hydrolyse internal peptide bonds on the amino side of hydrophobic residues [8,22].

The time course of the hydrolysis of THF and accumulation of its products is depicted in Fig. 2. After 90 min of incubation about 40% of the THF was hydrolysed. Incubation under the same conditions for 300 min resulted in over 75% hydrolysis of the peptide. Phosphoramidon (3.6 µM), a selective inhibitor of NEP [23], affords over 95% protection of THF (0.25 mM) against hydrolysis by NEP, even after prolonged incubation (5 h). Both the hydrolysis of THF by NEP into two distinct products and the protection provided by phosphoramidon were confirmed by t.l.c., with the use of standard THF and Phe-Leu samples for identification.

The kinetic parameters for the hydrolysis of THF by NEP, derived from a Lineweaver-Burk plot, were $k_{\text{cat}} = 19.7 \text{ s}^{-1}$ and $K_m = 83 \text{ µM}$ (Fig. 3).

Amino acid composition and sequence determination of the products of THF cleavage by NEP

Fractions eluted from h.p.l.c. columns after partial hydrolysis of THF by NEP were subjected to amino acid composition

Fig. 1. H.p.l.c. elution profile of THF hydrolysis by NEP

(a) THF (0.96 mM) was incubated with NEP (0.48 µg/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl at 37 °C. A 5 µl sample of the solution was injected into the h.p.l.c. system after 90 min incubation and heat inactivation of the enzyme. Peak II is the unchanged peptide and peaks I and III are the NEP cleavage products, identified as the N-terminal hexapeptide and the C-terminal dipeptide respectively. (b) 0.4 µl of THF (1.5 mg/ml). (c) 0.5 µl of Phe-Leu (1.0 mg/ml).

Fig. 2. Time course of the hydrolysis of THF by NEP

THF (0.90 mM) was incubated with NEP (0.48 µg/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl at 37 °C in the presence of 4 µM-bestatin. Samples taken at indicated times were injected into the h.p.l.c. system (10 µl) and areas were estimated by peak integration. ●, THF; ▲, Phe-Leu; □, hexapeptide.

Fig. 3. Kinetics of THF hydrolysis by NEP

The Figure shows a Lineweaver-Burk plot of the hydrolysis of 15–800 µM-THF incubated with NEP (0.24 µg/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl at 37 °C for 10 min. Data points are the average for two or three injections.

Table 1. Amino acid composition and sequence analyses of THF and its hydrolysis products

H.p.l.c. peak	Retention time (min)	Amino acid analyses	
		Composition* (molar proportions)	Sequence determination
I	5.00	Leu (1.0), Glu (1.2), Asp (1.1), Gly (1.3), Pro (1.1), Lys (1.0)	Leu-Glu-Asp-Gly-Pro-Lys
II	6.95	Leu (2.0), Glu (1.2), Asp (1.1), Gly (1.1), Pro (1.2), Lys (1.0), Phe (1.0)	Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu
III	7.67	Phe (1.0), Leu (1.0)	N.D.†

* Molar proportions of 0.1–0.2 were found for some other amino acids.
† Not determined.

Fig. 4. Effect of THF on the hydrolysis by NEP of Suc-Ala-Ala-Leu-NH-Np

The Figure shows Lineweaver-Burk plots of Suc-Ala-Ala-Leu-NH-Np hydrolysis. Data points were obtained from one to three experiments. ●, No inhibitor added; ■, 60 μM -THF; ▼, 120 μM -THF.

analyses and to sequence determination. As shown in Table 1, peak I is the *N*-terminal hexapeptide Leu-Glu-Asp-Gly-Pro-Lys, and peak III, whose mobility corresponds to that of a standard sample of Phe-Leu (Fig. 1c), is the *C*-terminal dipeptide Phe-Leu. Peak II is, as expected, unchanged THF. These findings confirm that the Lys⁶-Phe⁷ bond is the site of cleavage by NEP.

Kinetics of inhibition by THF

In order to establish further the action of NEP on THF, the ability of THF to inhibit the enzymic hydrolysis of the synthetic substrate Suc-Ala-Ala-Leu-NH-Np was assessed. The NEP concentration in these experiments was 12-fold lower than that employed in the experiments presented in Figs. 1(a) and 2, in order to minimize consumption of THF during hydrolysis of the substrate. This decrease in enzyme concentration could be readily accomplished because of the high sensitivity of the spectroscopic method used to assess enzyme activity [18]. As shown in Fig. 4, THF acts as a competitive inhibitor of the cleavage of Suc-Ala-Ala-Leu-NH-Np by NEP. The inhibition constant ($K_i = 97 \mu\text{M}$)

derived from these inhibition experiments is very similar to the K_m found ($K_m = 83 \mu\text{M}$).

DISCUSSION

The present study demonstrates that NEP efficiently cleaves the Lys⁶-Phe⁷ bond of THF. Analysis of the enzymic digest of THF by h.p.l.c. (Figs. 1a and 2) reveals that two peptide fragments are the products of this digestion. The identification of the products as the *N*-terminal hexapeptide and the *C*-terminal dipeptide was facilitated by analysing the elution profiles of the standard dipeptide Phe-Leu and of THF itself (Figs. 1b and 1c), and confirmed by amino acid analyses and sequence determinations (Table 1). The k_{cat} and K_m values for the hydrolysis of THF by NEP are 19.7 s^{-1} and $83 \mu\text{M}$ respectively (Fig. 3). In support of the analysis of the products of the enzymic digestion and of the kinetic studies, THF was found to act as a good competitive inhibitor of the action of NEP on the synthetic substrate Suc-Ala-Ala-Leu-NH-Np (Fig. 4).

The findings of a high turnover rate for NEP acting on THF, in the 10–100 molecules/s range, together with an affinity of THF for NEP of the order of 10^4 M^{-1} is of particular interest, in view of the possible roles that NEP might play in the immune system. NEP is present in bone marrow [3], lymph nodes and spleen [1], as well as on human neutrophils [10] and acute-lymphoblastic-leukaemia lymphocytes [3]. It was also found on bone-marrow-stroma-derived cell lines [24]. The enzyme has been implicated in the inactivation and metabolism of many peptides, including the enkephalins and Substance P [10,15]. These neuropeptides are also capable of influencing immune cell function [25]. Recently the ability of NEP to inactivate interleukin-1 β was reported [26]. Another cell-surface peptidase known to be present in the immune system is aminopeptidase N, also identified as the human myeloid plasma-membrane glycoprotein CD 13 [27,28].

Remarkably, THF and [Leu⁵]enkephalin have the same *C*-terminal dipeptide sequence Phe-Leu, and both peptides are acted upon by NEP to yield the same dipeptide. Furthermore, both the rate of hydrolysis and the affinity of [Leu⁵]enkephalin [15] and THF to NEP are similar. The values reported in this paper for THF represent a very high k_{cat}/K_m , in the 10^5 – $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ range.

It is now well accepted that a relatively small number of peptidases can act on a wide variety of peptides, depending on the tissue distribution of each enzyme, its localization, its specificity with regard to each peptide and its effective concentration [29,30]. NEP is an efficient endopeptidase known to be present on mammalian cell surfaces and is present in the immune system, suggesting a modulatory role on the activity of regulatory peptides acting in this system.

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