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# The effect of N-terminal acetylation and the inhibition activity of acetylated enkephalins on the aminopeptidase M-catalyzed hydrolysis of enkephalins<sup>☆</sup>

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## Abstract

High performance liquid chromatography and high performance liquid chromatography/electrospray ionization-mass spectrometry were used to study the effect of N-terminal acetylation and the inhibition activity of acetylated enkephalins on the aminopeptidase M (EC 3.4.11.2)-catalyzed hydrolysis of methionine (Met-enk) and leucine enkephalins (Leu-enk). Acetylation imparts a significant enhancement in the proteolytic stability of these two peptides. After 30 min of the reaction, <10% of both acetylated enkephalins was hydrolyzed. In an 8-h incubation period, only a maximum of 54% acetylated (Ac)-Met-enk and 38% Ac-Leu-enk was hydrolyzed.  $V_{\max}$  and  $K_m$  [infi] for the degradation of Ac-Met-enk were 1.4 nmol/min/50 ng and 2.2 mM, respectively. The corresponding values for the reaction of Ac-Leu-enk were 0.5 nmol/min/50 ng and 0.9 mM. Also, the aminopeptidase M activity on Met-enk can be inhibited in the presence of Ac-Met-enk, which acts as a mixed-type inhibitor with the inhibition constant ( $K_i$ ) of  $1 \times 10^{-3}$  M. © 1999 Elsevier Science Inc. All rights reserved.

**Keywords:** Methionine enkephalin; Leucine enkephalin; Acetylated methionine enkephalin; Acetylated leucine enkephalin; Aminopeptidase M; Proteolytic stability; Electrospray ionization mass spectrometry

## 1. Introduction

Characterization of opiate receptors in the early 1970s led to the knowledge of three families of opoid peptides: endorphins, dynorphins, and enkephalins. Each family is derived from a different precursor protein [15]. Enkephalins are biochemically synthesized from preproenkephalin A and binds preferentially to  $\delta$  opioid receptor [17]. Preproenkephalin A is a precursor of several opioid peptides, the most important being methionine enkephalin (Met-enk = YGGFM) and leucine enkephalin (Leu-enk = YGGFL). These two pentapeptides are found in various body tissues and are responsible for a variety of physiological activities in different tissues. They are potential candidates for analgesic drugs [18].

The pharmacological significance of these peptides, however, is diminished by the fact that they are rapidly degraded by endogenous enzymes before they can bind to their receptors

[4,12,14,19,21,22,27]. The primary route of inactivation is cleavage of the Tyr-Gly bond catalyzed by the soluble membrane-bound aminopeptidases (see Fig. 1). Other enzymes involved in the inactivation of enkephalins are dipeptidylaminopeptidase, angiotensin-converting enzyme, endopeptidase-24.11, and carboxypeptidases.

To enhance the biologic activity of enkephalins as analgesics, their proteolytic stability must be improved [13,18]. Such attempts include replacement of D amino acids for L amino acids, incorporation of unusual amino acids, and N-methylation of the amide linkages. Another innovative approach is the rational design of inhibitors for enkephalin degrading enzymes. Such compounds will prolong the lifetime of endogenously released enkephalins and may be devoid of the side-effects associated with narcotic drugs. Some of the compounds studied as inhibitors for aminopeptidases are thiorphan, amastatin, bestatin and its analogs, and kelatrophan and its analogs [2,23,24]. A recent study from our research group has demonstrated that phosphorylation of the tyrosine residue of Met-enk and Leu-enk leads to increased resistance against the aminopeptidase M (EC 3.4.11.2) activity [9].

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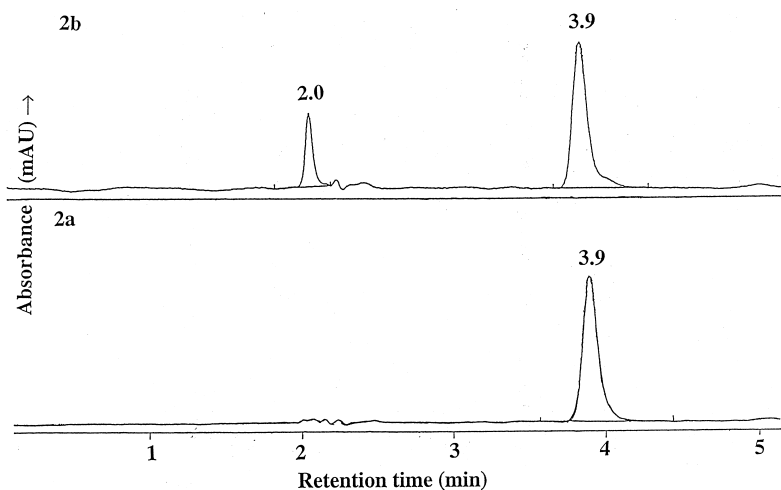


Fig. 2. HPLC chromatograms showing separation of the products of the reaction of aminopeptidase M with Ac-Met-enk (detection at 280 nm). The products were separated isocratically by using 20% acetonitrile in 10 mM ammonium acetate at a flow rate of 1.5 ml/min. (a) Incubation for a period of 10 min—no reaction products are seen, only Ac-Met-enk elutes at 3.9 min. (b) Incubation period of 1 h—only 20% of the substrate is hydrolyzed. The peaks eluting at 2.0 and 3.9 min are due to Ac-Tyr and Ac-Met-enk, respectively.

0.9, 2.0, and 3.0) also was studied under similar experimental conditions. The cross-over inhibition activity of acetylated enkephalins also was investigated using the same protocol. For degradation of Met-enk in the presence of Ac-Leu-enk, the [Ac-Leu-enk]/[Met-enk] ratio was adjusted to 2.0 and 3.0. For the hydrolysis reaction of Leu-enk, the [Ac-Met-enk]/[Leu-enk] ratio was also equal to 2.0 and 3.0.

## 2.5. Experiment 3

### 2.5.1. The type of inhibition

To determine the type of inhibition, the kinetic parameters  $V_{\max}$  and  $K_m$  were determined for the noninhibited reaction and  $V_{\text{app}}$  and  $K_{\text{app}}$  (app = apparent) for the inhibited reaction. For the noninhibited reaction, a portion (1.0 ml) of different concentrations of Met-enk (0.4–2.0 mM) was separately reacted with 50 ng of aminopeptidase M for an incubation period of 5 min. The experiment was repeated in the presence of 0.5 mM Ac-Met-enk, and the apparent kinetic constants ( $V_{\text{app}}$  and  $K_{\text{app}}$ ) were determined. The upper concentration of Ac-Met-enk was restricted to 0.5 mM because of solubility limitations.

## 2.6. Chromatography

Reversed-phase HPLC separation of the reaction products and nonreacted substrates was performed by using a HP1050 quaternary pumping system (Hewlett Packard) outfitted with a photodiode array ultraviolet-visible detector and ChemStation-based data system. The separated compounds were eluted isocratically at ambient temperature with Vydac C-18 (218TP54) column (250 × 4.6 mm inner diameter with 5- $\mu\text{m}$  particle size and 300- $\text{\AA}$  pore size). Separation of the products of enzymatic degradation of Ac-enkephalins and aminopeptidase M was optimized at the

flow rate of 1.5 ml/min by using a mobile phase of 20% acetonitrile in 10 mM ammonium acetate solution (pH 4.2). UV detection was carried out at 225 and 280 nm. Tyr and Tyr-containing products are detected at 280 nm [25].

In Experiment 2, the products were separated at the flow rate of 1.0 ml/min and the mobile phase composition of 12% acetonitrile in 10 mM ammonium acetate solution using the same column that was used in Experiment 1. For separation of the reaction products and the reactants of the Experiment 2 were used. Because of enhanced sensitivity, the ultraviolet absorbance at 225 nm was used to monitor the reaction products. Quantification of the amount of the unreacted Met-enk was based on a calibration curve constructed using a series of Met-enk solutions (0.1–2.4 mM).

## 2.7. Mass spectrometry

The separated reaction products were identified further by their mass-to-charge ( $m/z$ ) ratio by using a Micromass Platform II mass spectrometer (Micromass, Manchester, UK), operated in the ESI mode. The LC column was connected to the ESI probe by means of a flow splitter, which permitted only a fraction (200  $\mu\text{l}/\text{min}$ ) of the LC eluent to enter into the ESI source maintained at 180°C. Nitrogen was used both as nebulizing and drying gas at the flow rates of 15 l/h and 350 l/h, respectively. The sampling cone voltage was set to 30 V. These experimental parameters were optimized to minimize the formation of solvent clusters, which otherwise contribute significantly to the background chemical noise [28]. The MS data were acquired in the form of total ion current chromatograms by scanning the quadrupole in the positive-ion mode at a scan rate of 0.1s/scan and mass range of 145–625 Da. The instrument was controlled by a PC-based Masslynx data system.

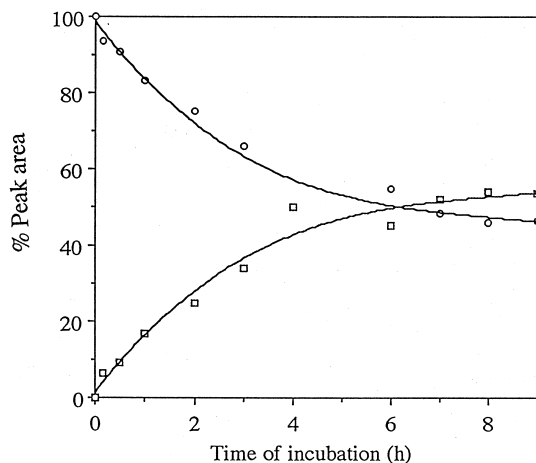


Fig. 3. The rate of disappearance of Ac-Met-enk (circles) and appearance of Ac-Tyr (squares). Ac-Met-enk was reacted with aminopeptidase M at 37°C and pH 7.02. % Peak area, the % peak area of Ac-Tyr or Ac-Met-enk relative to the sum of the peak areas of Ac-Tyr and Ac-Met-enk.

### 3. Results

#### 3.1. Reaction of Ac-Met-enk and Ac-Leu-enk with aminopeptidase M

Typical chromatograms illustrating the analysis of the reaction products of the reaction of Ac-Met-enk with aminopeptidase M are shown in Fig. 2. Similar chromatograms were obtained for the reaction of Ac-Leu-enk (not shown). The increase of the peak area of the Ac-Tyr (retention time, 2.0 min) was used as a marker of the progress of the reaction.

The progress of the hydrolysis reaction of Ac-Met-enk and Ac-Leu-enk is shown in Figs. 3 and 4, respectively. Almost 90% of both acetylated peptides remained intact after 30 min of the start of the reaction. A maximum of only

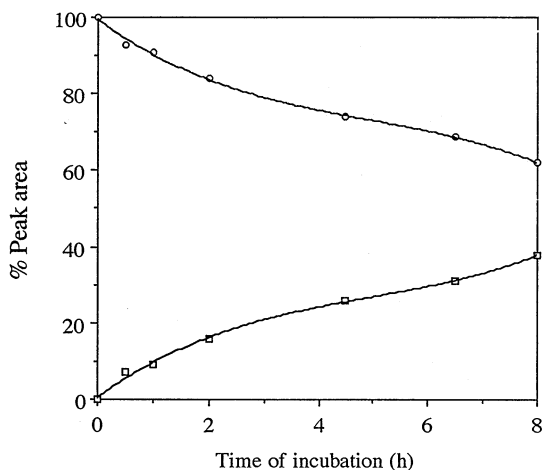


Fig. 4. The rate of disappearance of Ac-Leu-enk (circles) and appearance of Ac-Tyr (squares). Ac-Leu-enk was reacted with aminopeptidase M at 37°C and pH 7.02. %Peak area, the % peak area of Ac-Tyr or Ac-Met-enk relative to the sum of the peak areas of Ac-Tyr and Ac-Leu-enk.

Table 1

The effect of the increasing amounts of acetylated enkephalins on the degradation of nonacetylated enkephalins

[Ac-Met-enk]/ [Met-enk]	% Degradation	[AC-Leu-enk]/ [Leu-enk]	% Degradation
0.25	100.00 ± 0	0.90	100.00 ± 0
0.90	43.00 ± 2	2.00	98.00 ± 0.5
2.00	21.00 ± 9	3.00	94.00 ± 0.8

54% of Ac-Met-enk and 38% of Ac-Leu-enk were hydrolyzed at the end of 8-h incubation period. In contrast, unmodified Met-enk and Leu-enk are completely hydrolyzed by aminopeptidase M under identical experimental conditions within 1 min of the reaction (data not shown). The Hanes–Wolf plot, which is a plot of the ratio of [Met-enk]/(reaction-rate) against [Met-enk], was used to calculate the  $V_{max}$  and  $K_m$  values. Because of equal error distribution in both high and low concentration extremes, this plot is preferably used in obtaining precise estimates for these kinetic constants [5]. The values of the  $V_{max}$  and  $K_m$  for the degradation of Ac-Met-enk are found to be 1.4 nmol/min/50 ng and 2.2 mM, respectively. The corresponding values for the reaction involving Ac-Leu-enk are 0.5 nmol/min/50 ng and 0.9 mM, respectively.

#### 3.2. The inhibition activity of acetylated enkephalins on the aminopeptidase M-catalyzed hydrolysis of enkephalins

In contrast to the control experiment, in which the Met-enk is completely hydrolyzed within the incubation period of 10 min, intact Met-enk was detected in the presence of Ac-Met-enk (see Table 1). With increase in the ratio of [Ac-Met-enk]/[Met-enk], the extent of degradation of Met-enk (as measured by the peak area of Tyr in the HPLC chromatogram) was reduced considerably. At the ratio of 2.0, only 21% of the original amount of Met-enk was hydrolyzed as compared to complete hydrolysis in the absence of Ac-Met-enk. At 280 nm, only Tyr and Met-enk and Ac-Met-enk were detected at the retention times of 4.0, 9.3, and 11.4 min, respectively. At 225 nm, a few additional peaks corresponding to FM (at 4.8 min) and GGFM (at 6.2 min) also were detected.

By using HPLC/ESI-MS analysis, reaction products were identified further by their  $m/z$  ratio. In the control experiment, ions corresponding to Tyr ( $m/z$  182; at 4.0 min), FM ( $m/z$  297, at 5.0 min), and GGFM ( $m/z$  411, at 6.1 min) were detected (Fig. 5a). Met-enk ( $m/z$  574) and YGG ( $m/z$  296) were not detected. In the inhibited reaction mixture, molecular ions of Tyr, GGFM, Met-enk, and Ac-Met-enk ( $m/z$  616) were detected at 4.0, 6.1, 9.3, and 11.4 min, respectively (Fig. 5b). However, Ac-Tyr ( $m/z$  224) as well as YGG and FM were not detected.

The results of the degradation of Leu-enk in the presence of the varying amounts of Ac-Leu-enk also are included in Table 1. In this experiment, even at the [Ac-Leu-enk]/[Leu-



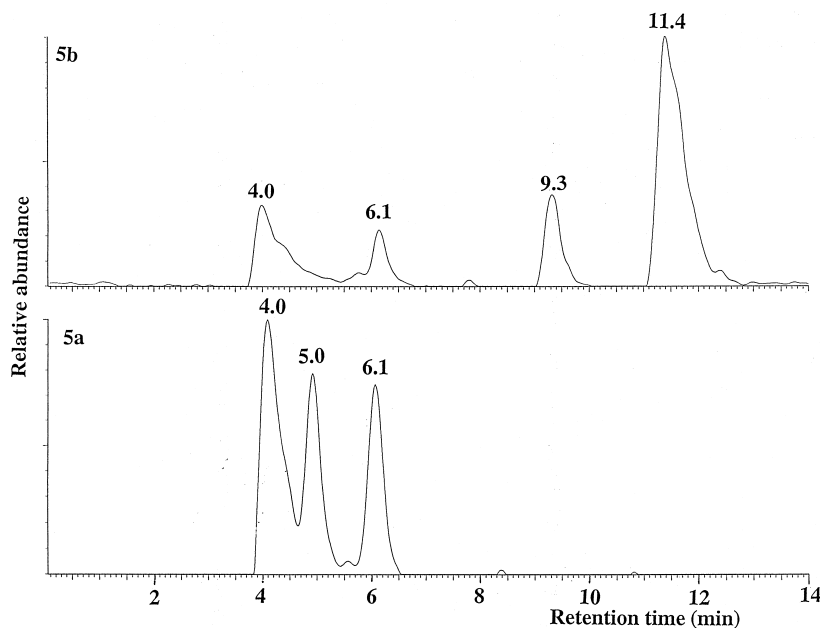


Fig. 5. The total ion current chromatograms obtained by on-line HPLC/ESI/MS. The reaction products were separated isocratically by using 12% acetonitrile in ammonium acetate at a flow rate of 1 ml/min. Only 200  $\mu$ l/min of the solvent was allowed to enter the ESI source. (a) The hydrolysis of Met-enk-only Tyr, FM, and GGFM were detected at retention times of 4.0, 5.0, and 6.1 min, respectively. Met-enk and YGG were not detected. (b) The hydrolysis of Met-enk in the presence of Ac-Met-enk- molecular ions of Tyr, GGFM, Met-enk, and, Ac-Met-enk detected at 4.0, 6.1, 9.2, and 11.3 min, respectively. Ac-Tyr, YGG, and FM were not detected.

enk] ratio of 3.0, only 6% of the original amount of Leu-enk remained intact. In the enzyme hydrolysis reactions of Leu-enk and Met-enk carried out in the presence of Ac-Met-enk and Ac-Leu-enk, respectively, both Leu-enk and Met-enk were hydrolyzed completely within the incubation period of 10 min.

### 3.3. The type of inhibition

The data for the noninhibited reaction and inhibited reactions are compared in Figs. 6 and 7. The Fig. 6a, which is the plot of reaction rate vs. the concentration of Met-enk, indicates that the reaction rate in the inhibited reaction is lowered. The kinetic constants for the noninhibited ( $V_{\max}$  and  $K_m$ ) and the inhibited ( $V_{\text{app}}$  and  $K_{\text{app}}$ ) reactions were determined by the Hanes–Woolf Plot. The data in Table 2 clearly indicate that there was a significant decrease in  $V_{\text{app}}$  and  $K_{\text{app}}$  compared with  $V_{\max}$  and  $K_m$ . As described above, the Hanes–Woolf Plot gives better estimates for  $V_{\max}$  and  $K_m$ . However, in the literature, the Lineweaver–Burk plot is used most commonly for the purpose of describing the types of inhibition. Therefore, to identify the type of inhibition, the Lineweaver–Burk plots (Fig. 7) were drawn from the values of  $V_{\max}$ ,  $K_m$ ,  $V_{\text{app}}$ , and  $K_{\text{app}}$  obtained from the Hanes–Woolf plot.

### 3.4. Statistical analyses

In Tables 1 and 2, the data are presented as  $\pm$  SD. The kinetic constants for the inhibited and noninhibited reac-

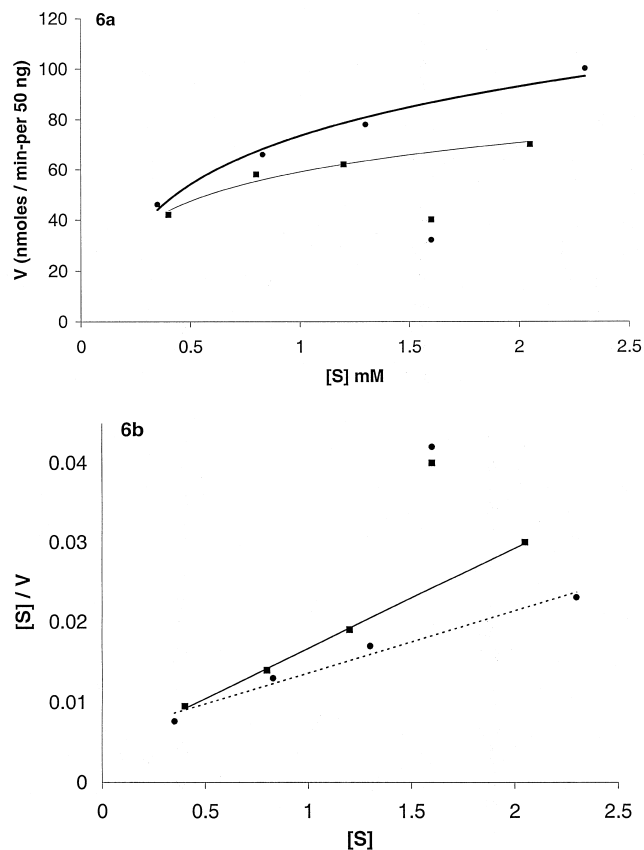


Fig. 6. Comparison of the Ac-Met-enk-inhibited reaction (filled squares) with the noninhibited reaction (filled circles).  $V$ , reaction rate in nmol/min/50 ng;  $[S]$ , [Met-enk] in mM. (a) Plot of the reaction rate vs. [Met-enk]. (b) The Hanes–Woolf plots for the inhibited and noninhibited reactions.

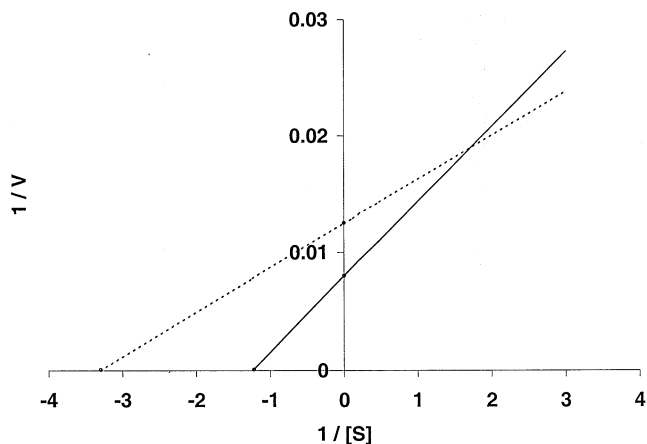


Fig. 7. The Lineweaver–Burk plots for the inhibited reaction (dotted line) and the noninhibited reaction (dark line). These plots were drawn based upon the  $K_m$  [infi] and  $V_{max}$  values obtained from the Hanes–Woolf plots in Fig. 6b.  $V$  and  $[S]$  are the same as in Fig. 6.

tions in Table 2 were calculated from the best fit straight line of the Hanes–Woolf plot disregarding the outliers at 1.6 mM substrate concentration. These results were compared by using one-tailed  $t$ -test at significance level of  $P < 0.01$ . Data were gathered by repeating each experiment at least twice.

#### 4. Discussion

In an effort to understand the role of N-terminal acetylation in enhancing the proteolytic stability of enkephalins, we have investigated the aminopeptidase M-catalyzed proteolysis of Ac-Met-enk and Ac-Leu-enk. As shown in Fig. 1, the first step of the aminopeptidase M-catalyzed hydrolysis of both these enkephalins will result in the formation of Ac-Tyr residue and the des-Ac-Tyr products, GGFM or GGFL, respectively. The predominant degradation product for both the substrates, however, is Ac-Tyr. The des-Ac-Tyr products were not detected perhaps because of the longer time interval (1 h) between two consecutive samplings. During this time interval, the initially formed GGFM (or GGFL) is hydrolyzed rapidly by sequential removal of the terminal amino acids. Therefore, the progress of the reaction was monitored based on the Ac-Tyr peak area. It is likely

Table 2  
Comparison of the kinetic constants calculated for the noninhibited reaction and the inhibited reaction

Kinetic constants	Noninhibited reaction <sup>a</sup>	Inhibited reaction <sup>b</sup>
$V_{max}$ nmol/min/50 ng	$130 \pm 9$	$80 \pm 5^*$
$K_m$ mM	$0.8 \pm 0.1$	$0.3 \pm 0.07^*$

<sup>a</sup> The Met-enk and aminopeptidase M reaction.

<sup>b</sup> The Met-enk and aminopeptidase M reaction in the presence of Ac-Met-enk.

\*  $P < 0.01$ .

that other amino acids, especially Phe, generated during hydrolysis of acetylated enkephalins may coelute with Ac-Tyr. However, it was observed by us using the standard samples that Phe is not detected at 280 nm (results not shown). Under these chromatographic conditions, only Ac-Tyr, Tyr, Met-enk, and Ac-Met-enk responded at 280 nm.

According to the results obtained in the Experiment 1, a significant proteolytic stability is demonstrated for Ac-Met-enk and Ac-Leu-enk against the activity of aminopeptidase M. The enhancement of the stability is greater than what had been observed previously by us due to phosphorylation of the Tyr residue [9]. This enhancement in stability due to acetylation is somewhat predictable because N-acetylation blocked the free amino terminal, which is considered an important site for aminopeptidase action [10].

Although N-terminal acetylation of enkephalins has imparted significant proteolytic stability, some enzymatic activity is still detected during the hydrolysis of Ac-Met-enk and Ac-Leu-enk. This observation led us to believe that Ac-Met-enk and Ac-Leu-enk may play the role of inhibitors in the degradation of enkephalins by competing for the aminopeptidase M activity. To test this hypothesis, we studied the enzymatic degradation of Met-enk and Leu-enk in the presence of their corresponding acetylated analogues (Experiment 2).

When acetylated and nonacetylated Met-enk were both present in the reaction mixture, it was necessary to use different chromatographic conditions than employed in the study of degradation of acetylated enkephalins. Chromatographic separation was optimized by using 12% acetonitrile in ammonium acetate buffer as the mobile phase at a flow rate of 1.0 ml/min. Under these conditions, both Tyr and Ac-Tyr elute at nearly the same retention time (4.0 min). However, the contribution of Ac-Tyr to the peak area of Tyr is negligible because degradation of Ac-Met-enk does not occur to any appreciable extent during the short incubation period (10 min) used in these experiments. This finding was further confirmed by HPLC/ESI-MS analysis of the reaction products. No Ac-Tyr was detected in the ion chromatogram. ESI-MS analysis of the reaction products also revealed the absence of the molecular ions of YG, YGG, and GG in the ion current chromatograms, indicating that the enzyme preparations used by us does not contain other enkephalin-degrading enzymes (Fig. 1) as impurities. It has been reported earlier that commercial preparations of kidney aminopeptidase M may be contaminated with neutral metalloendopeptidase [1]. If endopeptidase activity were present, it should have produced both YGG and FM. The absence of YGG molecular ion in the ESI-MS ion chromatogram rules out the possible contamination of aminopeptidase M with endopeptidases.

Decrease in the amount of degradation of Met-enk from 100% in the control experiment to 21% in the presence of Ac-Met-enk clearly indicates that Ac-Met-enk inhibits the aminopeptidase M-catalyzed hydrolysis of Met-enk. The absence of FM in this reaction mixture further supports this

inference. Inhibition of the aminopeptidase M activity also would affect the hydrolysis of subsequent N-terminal residues.

It was observed that Ac-Leu-enk is not as strong an inhibitor of the aminopeptidase M activity on the hydrolysis of Leu-enk as Ac-Met-enk is on the corresponding reaction of Met-enk. This observation is consistent with the fact that Ac-Leu-enk has lower affinity for aminopeptidase M compared to Ac-Met-enk (compare the  $V_{\max}$  values shown above). Also, no inhibition activity was found when Ac-Met-enk and Ac-Leu-enk were interchanged as inhibitors for the aminopeptidase M activity on Leu-enk and Met-enk, respectively. This intriguing observation can also be explained partially on the basis of relative affinities of Met-enk and Leu-enk for aminopeptidase M. Benter et al. [3] have reported that  $V_{\max}$  for the hydrolysis of the Tyr-Gly bond in human CSF is higher for Leu-enk (2.0 nmol/min/ml) than for Met-enk (400 pmol/min/ml).

The knowledge of the type of inhibition also may provide some explanation why Ac-Met-enk inhibits the hydrolysis of Met-enk and not Leu-enk. Inhibitors are classified as competitive, noncompetitive, uncompetitive, and mixed type [5,16,20]. Each type exhibits a characteristic deviation from the typical Lineweaver–Burk plot of the uninhibited reaction. Competitive inhibitors inhibit the enzyme action by binding to the same active site as the substrate. They increase the  $K_m$  but do not affect  $V_{\max}$ . With noncompetitive type inhibitors,  $V_{\max}$  is decreased but  $K_m$  remains unaffected. The noncompetitive inhibitors and the substrate bind to the enzyme reversibly, randomly, and independently at different sites. A pure uncompetitive inhibitor binds reversibly to the enzyme–substrate complex yielding an inactive enzyme–substrate–inhibitor complex. Its behavior is characterized by the decrease in both  $K_m$  and  $V_{\max}$ . The corresponding Lineweaver–Burk plot of the uncompetitive inhibition runs parallel to the plot of the noninhibited reaction. Unlike the competitive inhibition, the degree of inhibition increases with the increase in the substrate concentration in the uncompetitive inhibition because the enzyme is inactivated by the formation of an enzyme–substrate–inhibitor complex. If more than one of the above mechanisms are operative at the same time, the resulting inhibition is called mixed type, which is characterized by change in both  $K_m$  and  $V_{\max}$ .

Experiment 3 was performed to determine the type of inhibition and the corresponding inhibition constant. The data presented in Figs. 6 and 7 and Table 2 confirm that Ac-Met-enk can indeed inhibit aminopeptidase M-catalyzed hydrolysis of Met-enk. The presence of the uncompetitive inhibitor component is indicated clearly by the decrease in both  $K_m$  (from 0.8 to 0.3 mM) and  $V_{\max}$  (from 130 to 80 nmol/min/50 ng). The observation that the inhibition activity is prominent at higher substrate concentrations further supports this evidence. The Lineweaver–Burk plot (Fig. 7), however, does not obey the typical pattern (the plots of both inhibited and uninhibited reactions should be parallel) of a

pure noncompetitive inhibition. Therefore, the possibility of more than one mechanism exists. The deviation observed in Fig. 7 has been identified previously as due to a mixed type of inhibition [20], in which the inhibitor binds to the enzyme as well as to the enzyme–substrate complex. The inhibitor forms a dead-end complex by binding to the enzyme and reduces the total amount of enzyme molecules. It is likely that initial enzyme–Met-enk complex leads to favorable conformation in aminopeptidase M for binding to Ac-Met-enk. Because Ac-Met-enk does not behave like competitive inhibitors, it is unable to inhibit the aminopeptidase M-catalyzed hydrolysis of Leu-enk.

As explained in the experimental section, because of solubility constraints, highest [Ac-Met-enk] was limited to 0.5 mM. Therefore, 0.5 mM was considered as the limiting inhibitor concentration in calculating the inhibitor constant  $K_i$  [20]. The  $K_i$  for this system was found to be  $1.0 \times 10^{-3}$  M ( $\pm 0.7 \times 10^{-4}$  M). The threshold concentration of the substrate Met-enk above which inhibition is prominent was found to be  $\sim 0.5$  mM.

In conclusion, this study has confirmed that N-acetylation of enkephalins could enhance their stability against aminopeptidase M. Also, this study has revealed that Ac-Met-enk behaves as a mixed-type of inhibitor for the aminopeptidase M action on Met-enk.

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