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Endothelin 1 hydrolysis by rat kidney membranes

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Hydrolysis of endothelin 1 by rat kidney membranes was investigated using a reverse-phase HPLC and an automated gas-phase protein sequencer. Endothelin 1 was hydrolyzed into four major fragments which were detected by HPLC. Phosphoramidon, an inhibitor of neutral endopeptidase 24.11, almost completely suppressed the production of three fragments, but one fragment was not affected by the inhibitor. Analysis of N-terminal sequences of the degradation products revealed that the phosphoramidon-sensitive fragments were generated by cleavage at the Ser^5–Leu^6 bond of endothelin 1 that was identical with its cleavage site by purified rat endopeptidase 24.11, reported previously. The phosphoramidon-insensitive fragment was produced by cleavage at Leu^{17}–Asp^{18}, which was distinct from the sites by endopeptidase 24.11, but corresponded to that by a phosphoramidon-insensitive metallo-endopeptidase recently isolated from rat kidney membranes by us [(1992) Eur. J. Biochem. 204, 547–552]. Kinetic determination of endothelin 1 hydrolysis by the isolated enzyme yielded values of K_m=71.5 μM and k_{cat}=1.49 s^{-1}, giving a ratio of k_{cat}/K_m=2.08 × 10^4 $s^{-1}·M^{-1}$. The K_m value was much higher and the k_{cat}/K_m value was much lower than those for rat endopeptidase 24.11 reported previously. Thus, endopeptidase 24.11 appears to hydrolyze endothelin 1 more efficiently than the isolated enzyme does. Both enzymes may play physiological roles in the metabolism of endothelin 1 by rat kidney membranes in vivo.

Endothelin 1; Kidney membrane; Metallo-endopeptidase; Endopeptidase 24.11; Peptide hydrolysis; Rat kidney

1. INTRODUCTION

Recent evidence has revealed the importance of kidney membrane metallo-endopeptidases in the metabolism of biologically active peptides. Neutral endopeptidase 24.11 is one of the well-characterized renal enzymes and is known to participate in the metabolism of some peptide hormones [1,2]. For instance, this enzyme efficiently hydrolyzes atrial natriuretic peptide (ANP) in vitro [3–5], and the physiological importance of endopeptidase 24.11 in ANP metabolism has been further shown by in vivo studies in animals and man [6–8] in which administration of specific inhibitors of the enzyme caused increases in circulating ANP concentration with a concomitant augmentation in its physiological actions such as diuresis and natriuresis.

In contrast, very little is known about the metabolism of endothelin 1, a 21-amino acid peptide with a powerful vasoconstricting activity [9]. Several recent studies, however, suggest that renal endopeptidase 24.11 is also involved in the metabolism of endothelin 1 [10–12]. Membrane fractions from kidney homogenates contained a potent hydrolytic activity for endothelin 1 which was strongly inhibited by phosphoramidon, an inhibitor of endopeptidase 24.11 [10]. Purified rat and bovine endopeptidase 24.11 were shown to cleave endothelin 1 initially at the Ser^5–Leu^6 bond, followed by cleavage at Asp^{18}–Ile^{19}, in a fashion inactivating the peptide [11,12]. As there have been few studies on the mechanisms involved in endothelin 1 degradation by kidney membranes, however, it is still unclear whether endopeptidase 24.11 is the only degrading enzyme integrated in kidney membranes to act on the metabolism of endothelin 1 molecules. Indeed, a metallo-endopeptidase recently purified to homogeneity from rat kidney membranes by us [13] has the ability to hydrolyze various peptide hormones such as endothelin 1, ANP and parathyroid hormone in vitro [14], and seems to have the potential to metabolize endothelin 1 and other biologically active peptides in kidneys in vivo. The enzyme hydrolyzed endothelin 1 in a limited fashion at the Leu^{17}–Asp^{18} bond in vitro [14], which was clearly distinct from the sites cleaved by endopeptidase 24.11. In addition to the cleavage site of endothelin 1, this isolated metallo-enzyme was also distinguished from endopeptidase 24.11 by being insensitive to phosphoramidon and in having an oligomeric structure with a molecular weight of the native form of 250 kDa and of its subunits of 88 kDa [13].

In this work, to further clarify the mechanism of endothelin 1 degradation by kidney membranes, we analysed the degraded fragments using a reverse-phase HPLC and an automated gas-phase protein sequencer, and found that besides endopeptidase 24.11 a new enzyme, isolated by us from the kidney membrane fraction [13,14], was involved in the metabolism of endothelin 1. Both enzymes may be physiologically important in the renal metabolism of endothelin 1 in vivo.

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2. MATERIALS AND METHODS

2.1. *Materials*

Endothelin 1, phosphoramidon and amastatin were obtained from the Peptide Institute, Osaka, Japan. All other chemicals were of the highest grade commercially available.

2.2. *Preparation of rat kidney membranes*

Rat kidney membranes were prepared according to the method of Stephenson and Kenny [15].

2.3. *Hydrolysis of endothelin 1 and peptide-sequence analyses*

20 μM endothelin 1, 5 μg rat kidney membranes and 100 μM amastatin, an inactivator of aminopeptidase, were dissolved in 20 mM Tris-HCl buffer, pH 8.0, in a total volume of 250 μl. Samples were incubated at 37°C for 16 h and reactions were terminated by adding 12.5 μl 100% (by vol.) acetic acid. The mixtures were then fractionated by HPLC on an ODS 120T reverse-phase column (C_{18}; 4.6 × 250 mm; Toyo Soda, Tokyo, Japan) with a linear gradient of 0–45% acetonitrile in 0.1% trifluoroacetic acid for 40 min at a flow rate of 1 ml/min. Elution was monitored by absorbance at 215 nm. Degradation products were collected in test tubes and freeze-dried. The amino acid sequences of these samples were determined with an Applied Biosystems model 470A gas-phase sequencer and model 120A HPLC analyzer system.

2.4. *Kinetic determination for endothelin 1 hydrolysis by a metallo-endopeptidase isolated from rat kidney membranes*

Kinetics for endothelin 1 hydrolysis by a metallo-endopeptidase recently isolated from rat kidney membranes [13] were measured by quantitative HPLC analysis of reaction mixtures. The incubation mixture (volume 250 μl) contained 2.17 nM (48 ng) rat enzyme and 5–80 μM endothelin 1 in 20 mM Tris-HCl buffer, pH 8.0. Samples were incubated at 37°C for 2 h and the reactions were stopped by adding 12.5 μl 100% (by vol.) acetic acid. The samples were then analyzed by HPLC as described above. Product peak areas were converted to concentration by defining the areas measured after complete hydrolysis as equal to the initial substrate concentration. Hydrolysis rates were assessed by the appearance of the products/unit time. Kinetic constants were calculated using the Michaelis–Menten equation and the linear transform methods of Lineweaver–Burk. The values for k_{cat} were calculated by assuming a catalytic subunit of 88 kDa. Three separate kinetic studies were performed using different enzyme preparations.

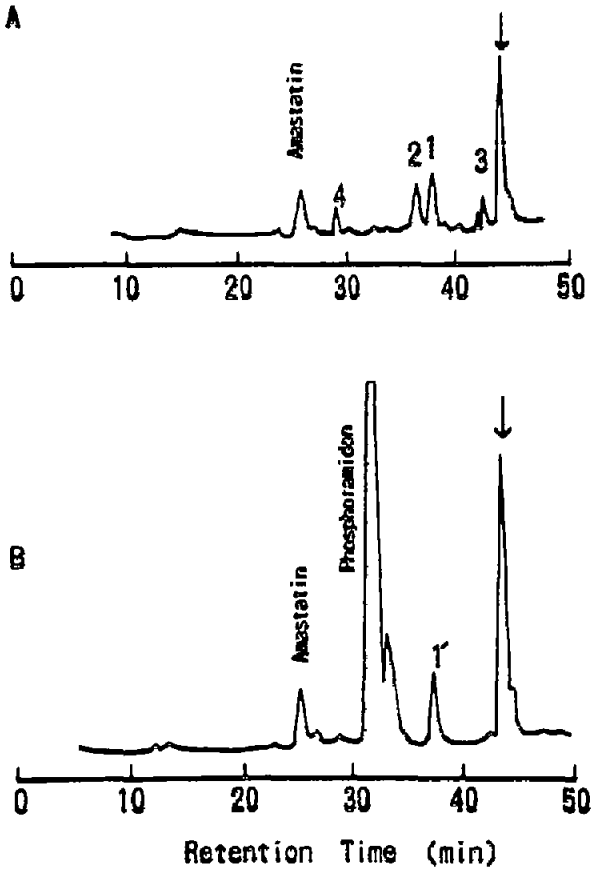

Fig. 1. HPLC analysis of peptide products formed by incubation of endothelin 1 with rat kidney membranes. Endothelin 1 (20 μM) was incubated with 5 μg kidney membranes in 20 mM Tris-HCl, pH 8.0, for 16 h at 37°C in the absence and presence of 100 μM phosphoramidon (A and B, respectively). Then, each sample was fractionated by HPLC as described in Materials and Methods. The product peaks are numbered in order of yield, and numbers correspond to those in Table I and Fig. 2. Arrows indicate the retention times of intact endothelin 1.

3. RESULTS AND DISCUSSION

Fig. 1A shows the HPLC pattern of endothelin 1 and its fragments after 16 h incubation with rat kidney membranes. Endothelin 1 was hydrolyzed by the membranes to four major degradation products (designated peaks 1–4) which were detected by their ultraviolet absorbance at 215 nm. In the presence of 100 μM phosphoramidon, peaks 2–4 disappeared on the HPLC profile, whereas peak 1 was unaffected and clearly eluted as peak 1′ at the corresponding retention time (Fig. 1B). The individual peak fractions shown in Fig. 1A and B were collected and their amino acid sequences were determined in an automated gas-phase sequencer (Table I). The sites of hydrolysis predicted from these analyses are shown in Fig. 2. The results showed that peak 1 and peak 1′, both insensitive to phosphoramidon, were identical and consisted of a mixture of Asp-Ile-Ile-Trp and the N-terminal fragment of endothelin 1, indicating that these fragments were generated by cleavage at the Leu^{17}–Asp^{18} bond. Peaks 2–4, all sensitive to phosphoramidon, were the N-terminal fragment of endothelin 1 (peak 2) or the products by cleavage at the Ser^{5}–Leu^{6} bond (peaks 3 and 4).

A metallo-endopeptidase recently isolated from rat kidney membranes by us [13] is insensitive to phosphoramidon and cleaves endothelin 1 in a limited fashion at the Leu^{17}–Asp^{18} bond [14]. In contrast, another metallo-endopeptidase from rat kidney membranes, endopeptidase 24,11, is known to be sensitive to phosphoramidon [1,2] and to cleave the hormone at the Ser^{5}–Leu^{6} and Asp^{18}–Ile^{19} bonds [11]. Therefore, the results presented above strongly suggest that peaks 1 and 1′ were generated by our newly isolated metallo-endopeptidase, whereas peaks 2–4 were produced by endopeptidase 24,11.

Endothelin 1 is supposed to be an important physiological substrate for rat endopeptidase 24,11 since the

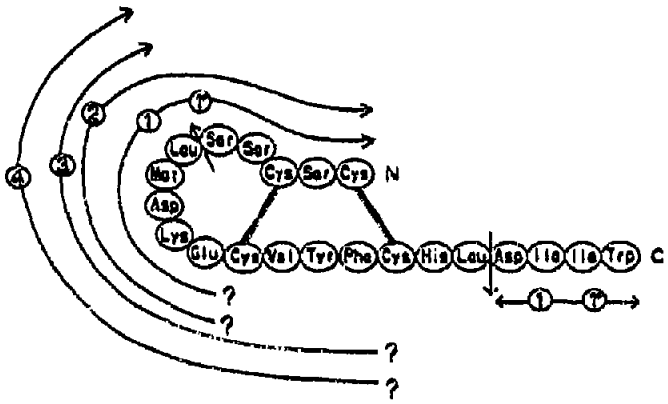

Fig. 2. Identities of the products formed by incubation of endothelin 1 with rat kidney membranes. The numbered peptides corresponding to the HPLC peaks in Fig. 1 were identified by determining amino acid sequences in an automated gas-phase sequencer. Arrows indicate the bonds attacked by the enzyme.

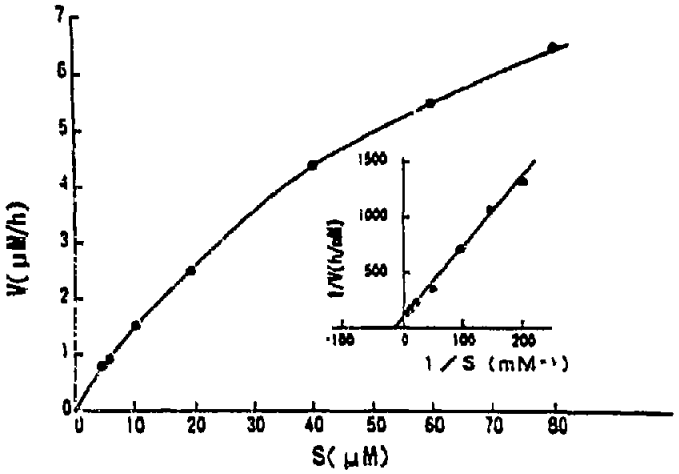

Fig. 3. Dependence of velocity of hydrolysis by a recently isolated metallo-endopeptidase on endothelin 1 concentration. Velocities for hydrolysis of various concentrations of endothelin 1 by a recently isolated rat metallo-endopeptidase were determined by quantitative HPLC analysis as described in Materials and Methods. A Lineweaver-Burk double reciprocal plot of the same data is shown in the inset.

K_m value for endothelin 1 is amongst the lowest reported for this enzyme (K_m=2.3 μM, k_{cat}=2.19 s^{-1}, k_{cat}/K_m=9.52 × 10^5 $s^{-1}\cdot M^{-1}$) [11]. To assess the potency of our isolated enzyme in endothelin 1 hydrolysis, we determined kinetic parameters for endothelin 1 hydrolysis by our enzyme and compared the obtained values with those by endopeptidase 24.11. As shown in Fig. 3, kinetic constants were calculated using the Michaelis–Menten equation and the linear transform methods of Lineweaver–Burk. Graphic analysis yielded values of K_m=71.5 μM, k_{cat}=1.49 s^{-1} (V_{max}=3.23 nM·s^{-1}; [enzyme]=2.17 nM at the subunit molecular mass of 88 kDa), giving a ratio of k_{cat}/K_m=2.08 × 10^4 $s^{-1}\cdot M^{-1}$. This K_m value by our enzyme for endothelin 1 is about 30-fold higher and the k_{cat}/K_m is about 50-fold lower than those for endopeptidase 24.11, respectively. Thus, taken together with the findings that three of the four peaks shown in Fig. 1A were the products by endopeptidase 24.11, this enzyme seems to metabolize endothelin 1 more efficiently than does our isolated enzyme from rat kidney membranes.

In conclusion, this study indicates that endopeptidase 24.11 and our isolated rat metallo-endopeptidase, both as integrated membrane proteins, participate in the metabolism of endothelin 1 by rat kidney membranes. Thus, both enzymes may be physiologically important, and further studies are needed to elucidate their roles in the renal metabolism of endothelin 1 in vivo.

Table 1

Amino acid sequences of degradation products of endothelin 1

Peak	Amino acid sequence	Cleavage site
1	Asp-Ile-Ile-Trp	Leu^{17}–Asp^{18}
	Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu	N-terminal
2	Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-	N-terminal
3	Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-	Ser^5–Leu^6
4	Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-	Ser^5–Leu^6
1′	Asp-Ile-Ile-Trp	Leu^{17}–Asp^{18}
	Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-	N-terminal

The individual peak fractions, as designated in Fig. 1, were collected and their amino acid sequence determined in an automated gas-phase protein sequencer as described in Materials and Methods.

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