Deamidase cleaves ester and peptide bonds in various substrates and deamidates protected COOH-termino-
amic acids. It preferentially hydrolyzes peptides
which contain hydrophobic amino acids in the P1, P1'
and/or P1' position. Because the COOH-terminal end
of endothelin I contains the hydrophobic sequence
-Ile19-Ile20-Trp21-OH, we investigated whether human
deamidase, purified from platelets, could inactivate
this peptide. We found that deamidase readily cleaved
off Trp21 with an acid pH optimum, a K_m of 22 μM, a
k_cat of 1454 min⁻¹, and a k_cat/K_m of 68 μM⁻¹ min⁻¹. We
also found the enzyme to be present in target cells of
endothelin I, in vascular smooth muscle cells. Extracts
of cultured vascular smooth muscle cells cleave both
the synthetic fluorescent substrate 5-dimethylaminon-
aphthalene-1-sulfonyl(Dns)-Phe-Leu-Arg and endo-
thenin I by releasing the COOH-terminal amino acid.
The reaction was inhibited by diisopropyl fluorophor-
phate, benzylxocarbonyl-Gly-Leu-Leu-Phe-CH&l was from Enzyme Systems Products (Livermore,
CA).

EXPERIMENTAL PROCEDURES

MATERIALS—5-Dimethylaminonaphthalene-1-sulfonyl-l-phenylala-
nyl-l-leucyl-l-arginine (Dns-Phe-Leu-Arg) was synthesized by cou-
ping Dns-Phe to Leu-Arg using standard techniques (9). Deamidase
was purified from human platelets as described (4). Trans-epoxysuc-
cinyl-l-leucylamido-(4-guanidino)-butane (E64) and other laboratory
reagents were obtained from Sigma, and benzylxocarbonyl (Z)-Gly-
Leu-Phe-CH&l was from Enzyme Systems Products (Livermore, CA).

Enzyme assays—The activity of the deamidase was determined
with ET1 substrate by separating and quantitating the products in
a final supernatant of homogenized cell preparations as enzyme
source, ET1 (8.6 pM, 100,000 x g final super-
натant of the homog-
eganized smooth muscle cells was 2.1 μmol/h/mg and
3.1 μmol/h/mg for Dns-Phe-Leu-Arg. Thus, smooth
muscle, platelets, and many other tissues which con-
tain the deamidase can inactivate endothelin by cleav-
ing the COOH-terminal tryptophan.

Endothelin I (ET1)² belongs to a group of peptides which
evert a potent and long lasting stimulation of vascular smooth
muscle that can elevate the blood pressure (1, 2). ET1 and its
congugers also contract intestinal and pulmonary smooth
muscles, affect cardiac and renal function, and are implicated
in the regulation of transmembrane signaling and gene expres-
sion (3). All mature endothelins contain 21 amino acids,
including 4 cysteine residues linked by two intramolecular
disulfide bonds and the unusually hydrophobic COOH-ter-
minal end of Ile19-Ile20-Trp21 (3).

Recently, we isolated and purified an enzyme from human
platelets which cleaves COOH-terminal free or protected
amino acids (4). It also deamidates peptides such as tachyki-
nins by converting the COOH-terminal Met-NH₂ to Met-OH.
Thus, it was called deamidase, but the enzyme is identical
with the so-called lysosomal protective protein (5, 6) and has
many properties in common with cathepsin A (4). The dea-
midase activity of the homogeneous protein has a neutral pH
optimum, while the carboxypeptidase-type action is more
effective at an acidic pH. The enzyme preferentially cleaves
substrates where the P1 residue is a hydrophobic amino acid.
Since our best synthetic substrates employed to assay the
enzyme have hydrophobic amino acids in either the P1 and
P1' or P1, P1', and P1' positions² (4), we tested ET1 as a substrate.
The high potency and widespread effects of ET1 require tight
control of its bioavailability, and that usually includes enzy-
matic degradation. Since the removal of the Trp from the
hydrophobic COOH terminus of ET1 terminates the vaso-
constrictor activity (7, 8), we hypothesized that this peptide
could play a role in the inactivation of ET1. Indeed, we found
that the deamidase inactivated ET1 by removal of Trp².

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² The abbreviations used are: ET1, endothelin I; HPLC, high
performance liquid chromatography; Dns, 5-dimethylaminonaph-
thalene-1-sulfonyl; E64, trans-epoxysuccinyl-l-leucylamido-
(4-guanidino)-butane; Z, benzylxocarbonyl; DFP, diisopropyl
fluorophosphate; PCMS, p-chloromercuribenzenesulfonate; MES, 2-
(N-morpholino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-l-
piperazineethanesulfonic acid; EGTA, ethylenediamine(oxy-
ethylennitritro)tetraacetic acid.
**RESULTS**

As established by HPLC, purified deamidase metabolized ET1 (8.6 μM) at a rate of 6.86 μmol/min/mg at pH 5.5 and 23 °C. The rate decreased at higher pH values to 2.32 μmol/min/mg at pH 6.25 and 0.36 μmol/min/mg at pH 7.0. The reactions were run at 23 °C because the purified enzyme is somewhat unstable at pH values >5.5 at 37 °C. The cleavage was not due to a putative contaminant in the purified deamidase preparation, because it was inhibited by three inhibitors with different modes of action. We previously showed that these compounds inhibit the enzyme with other substrates (4). Thus, ET1 hydrolysis at pH 5.5 or 7.0 was inhibited 98 or 100% with 1 mM DFP, 97 or 100% by 60 μM Z-Gly-Leu-Phe-CH₂Cl, and 78 or 100% by 1 mM p-chloromercuribenzenesulfonate (PCMS).

HPLC analyses of the reaction mixtures revealed the appearance of a new peak increasing in size with the length of incubation and proportional to the decrease in size of the substrate ET1 peak (Fig. 1). The metabolite peak did not appear or was greatly reduced in the presence of the deamidase inhibitors mentioned above (DFP, Z-Gly-Leu-Phe-CH₂Cl, or PCMS). In order to unequivocally identify this new product peak, in two experiments a higher concentration (35 μM) of ET1 was incubated with the deamidase, and the new peak and the peak coeluting with the unhydrolyzed ET1 substrate peak were collected. Amino acid analysis of the collected fractions showed that the new peak contained all the amino acids in the same molar ratios as the ET1 standard, with the important exception of tryptophan, which was missing. Consequently, because ET1 contains only a single COOH-terminal tryptophan (1), the deamidase cleaves the COOH-terminal amino acid from ET1. The peak which coeluted with the ET1 standard was indeed ET1 since it contained all the amino acids, including Trp²¹, as determined by amino acid analysis.

A third, less prominent peak was detected, but only with the higher concentration (35 μM) of ET1 (Fig. 1). The size of this peak also depended on incubation time and was reduced in the presence of inhibitors. The peak had the same elution time as a tryptophan standard and increased when the sample was spiked with authentic tryptophan. Thus, this peak contained the product, Trp²¹, resulting from the enzymatic hydrolysis of ET1 by deamidase.

Kinetic constants were determined for purified deamidase using ET1 as substrate at pH 5.5 and 23 °C. The Kₘ was 22 μM, and the Vₘₐₓ was 28 μmol/min/mg, giving a Kₘ/Vₘₐₓ of 1454 min⁻¹ and a kₐᵦ/Kₘ of 58 μM⁻¹ min⁻¹.

Because smooth muscle is a target tissue for endothelin, where it causes a long lasting contraction, we tested the final supernatant of homogenized cultured rat vascular smooth muscle cells for deamidase activity. The hydrolysis of Dns-Phe-Leu-Arg was measured fluorometrically, and ET1 inactivation was determined by HPLC. The final supernatant (S₆) of vascular smooth muscle cells hydrolyzed Dns-Phe-Leu-Arg at a rate of 3.1 μmol/h/mg at pH 5.5 and 0.8 μmol/h/mg at pH 7.0. ET1 was hydrolyzed at a rate of 2.1 μmol/h/mg at pH 5.5 and 0.3 at pH 7.0. Inhibition studies indicated that the activity in this fraction of the cells was due to deamidase. The reaction was not inhibited by o-phenanthroline (1 mM), the inhibitor of metalloproteases, or by the catheptic enzyme inhibitor E64 (100 μM), but the hydrolysis was almost completely abolished (95–100%) by DFP (1 mM) and Z-Gly-Leu-Phe-CH₂Cl (100 μM, Table I). The inhibition pattern was the same with both ET1 and the short synthetic substrate Dns-Phe-Leu-Arg (Table I).

The inactivation of the biological effects of ET1 by deamidase was observed in bioassay experiments on the isolated guinea pig ileum. Here, incubation of 0.2 pmol of deamidase with 100 nmol of ET1 at 37 °C for 45 min at pH 5.5 abolished 70% of the contractile effect of the peptide.

**DISCUSSION**

ET1 is released from a 38-amino acid proendothelin (or big endothelin) by the cleavage of the Trp²¹-Val²² bond. Three enzymes have been described to activate ET1; two of the more active ones are intracellular aspartic proteases with an acidic pH optimum. The third is a neutral metalloprotease. These converting enzymes are also present in vascular smooth muscle cells (12–18).

In spite of numerous publications on the variety of biological actions of endothelins, such as the long lasting vasoconstriction (1, 2, 18) and even on the cloning of its receptors (2, 19), there is a paucity of information on its enzymatic metabolism. Two recent publications reported the hydrolysis of ET1 by purified neutral endopeptidase 24.11 or enkephalinase in vitro (20, 21). This enzyme cleaves a variety of other peptide substrates including hypotensive peptides such as substance P and bradykinin, opioid peptides such as enkephalins, and atrial natriuretic factor (22). Interestingly, the enzymatic conversion of big endothelin to endothelin is inhibited in vivo.
by the neutral endopeptidase inhibitor, phosphoramidon, probably by blocking another enzyme (12–18). The reported \( K_{m} \) of ET1 with bovine kidney neutral endopeptidase is somewhat higher than the one we obtained with the human deamidase (30 versus 22 \( \mu \)M), but the \( K_{m} \) is lower (2.8 \( \mu \)M) with rat neutral endopeptidase (20, 21). However, the turnover number \( (K_{cat}) \) for ET1 with deamidase is much higher (1454 min\(^{-1}\)) than that reported for rat neutral endopeptidase (131 min\(^{-1}\)) (21). Based on the kinetic constants, deamidase would hydrolize 0.1 mM ET1 at a rate of 23 \( \mu \)mol/min/mg, which is about 10–25 times faster than rates we found (at pH 5.5) for other biologically active substrates such as angiotensin I, bradykinin, oxytocin, or substance P (4).

Although the deamidase used here is present in many tissues, it was purified from human platelets. The enzyme, as we showed, is identical with the so-called lysosomal protective variety of COOH-terminal free or protected amino acids, for biologically active substrates such as angiotensin I, bradykinin, oxytocin, or substance P (4).

Although these studies have been carried out in vitro, deamidase could very well be involved in the inactivation of endothelin in vivo. The long lasting pressor effects of injected endothelin indicate the peptide is not rapidly metabolized by blood-borne enzymes or plasma membrane-bound peptidases on endothelial cells. Indeed, a major endothelial cell peptidase, angiotensin I-converting enzyme, does not metabolize endothelin (20), and the only peptidase identified which does inactivate it, neutral endopeptidase 24.11, has a very low activity on plasma membranes of endothelial cells or in blood plasma under normal circumstances (29, 30). Thus, endothelin actions may be regulated by receptor-mediated endocytosis as shown for other peptide hormones (31). In this pathway, peptide-receptor complexes are endocytosed into coated vesicles which fuse with prelysosomal or lysosomal vesicles where the peptide ligand is degraded. In some cases, the receptor is recycled while in other cases it is also degraded. The lysosomal localization of deamidase and the rapid inactivation of ET1 by the enzyme make it an ideal candidate for this type of reaction.

In addition, deamidase is released from platelets by thrombin (4) and could thereby regulate endothelin levels at sites of platelet activation and aggregation such as in inflammation or at atherosclerotic plaques. This ability to affect vasoconstrictor activity at these sites is of obvious importance. Furthermore, lysosomal enzymes, for example cathepsins B, G, and L (32–35), can become associated with cell membranes, and in this way deamidase may contribute to the regulation of endothelin levels both in a soluble, released form and as an extracellular, membrane-bound enzyme.

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