A Mechanism-Based Inactivation Study of Neutral Endopeptidase 24.11

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The mechanism-based inactivation of human neutral endopeptidase 24.11 (NEP) was studied with *N*-[(*R*)-2-benzyl-5-cyano-4-oxopentanoyl]-L-phenylalanine (1) and its peptidic analogue, *N*-[*N*-(cyanoacetyl)-L-phenylalanyl]-L-phenylalanine (2). While both these active-site-directed molecules inactivate NEP, the related angiotensin-converting enzyme (ACE) is only inactivated by compound 2 [Ghosh et al. *J. Med. Chem.* **1992**, *35*, 4175-4179]. The selectivity in inactivation was addressed further by a comparative study of the interaction of compounds 1 and 2 with five other zinc proteases. The selective inactivation of NEP observed with the ketomethylene compound 1 suggests that the active site of NEP is less discriminating in its requirements for binding such substrate analogues as compared to ACE, a characteristic that may be exploited for designing specific mechanism-based inactivators for NEP. It is proposed that the inactivation is a result of NEP-catalyzed formation of ketenimine intermediates, which are subsequently trapped by an active-site nucleophile.

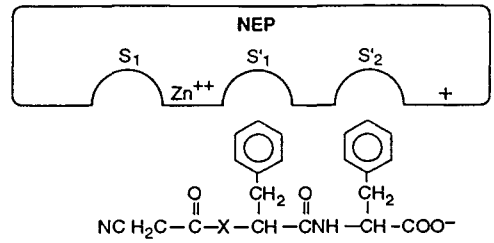
Introduction

Neutral endopeptidase 24.11 (NEP, EC 3.4.24.11) is a membrane-bound zinc metalloenzyme that cleaves a variety of bioactive peptides at the amino side of hydrophobic amino acid residues.[1] NEP plays an important role in the *in vivo* inactivation of neurotransmitters, Leu-enkephalin and Met-enkephalin, and atrial natriuretic factor (ANF), a hormone involved in the regulation of fluid balance and blood pressure.[2] Because of the biological significance of NEP, there has been considerable effort in designing inhibitors for the enzyme which may have potential application as antinociceptive and antihypertensive agents.[2,3]

The emphasis of our research has been on the development of mechanism-based inactivators for human NEP. The approach for these compounds is based on the observation that closely related zinc proteases, such as carboxypeptidase A (CPA) and angiotensin-converting enzyme (ACE) catalyze deprotonation of ketonic substrate analogues.[4,5] This characteristic was recently exploited for the design of specific mechanism-based inactivators for these enzymes, wherein it was proposed that enolization of α-cyanomethyl ketone or *N*-cyanoacetylamide substrates unmasks transient ketenimine intermediates, which then trap an active-site nucleophile.[6,7] We were interested in exploring whether the deprotonation/isomerization reaction is a general feature shared by other members of zinc protease family of enzymes. Furthermore, we were guided by the rationale that a mechanism-based inactivation approach would afford high selectivity in targeting specific enzymes. We report here that the general strategy for inactivation of zinc metalloproteases by the type of mechanism-based inactivators developed by us is indeed applicable for NEP, and that one of the inactivators shows high selectivity for the enzyme.

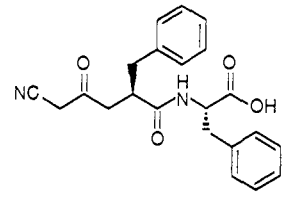
Results and Discussion

The design of *N*-[(*R*)-2-benzyl-5-cyano-4-oxopentanoyl]-L-phenylalanine (1) and its peptidic analogue, *N*-[*N*-(cyanoacetyl)-L-phenylalanyl]-L-phenylalanine (2) was based on the classical model for the active site of zinc proteases[8] (Figure 1). The following structural elements



1 X = CH_2
2 X = NH

Figure 1. Hypothetical model of the active site of NEP and of the binding of the inactivators 1 and 2.



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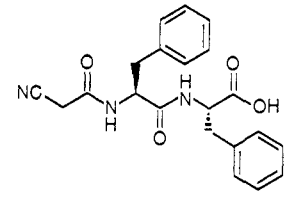

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were envisioned for binding NEP and for the enzyme's inactivation: (i) a C-terminal carboxylate group for ion pairing with the active site Arg 102,[9] (ii) a hydrophobic P'_1 residue based on the strong preference for aromatic or large hydrophobic residues in the enzyme's S'_1 subsite,[10] and (iii) incorporation of an α-cyanomethyl moiety, based on the precedent reported for CPA and ACE inactivation,[6,7] for the requisite NEP-catalyzed isomerization of compounds 1 and 2 to provide the electrophilic ketenimine intermediates. Preliminary inactivation studies established that Phe was the preferred P'_2 residue compared to Gly and Ala (data not shown). Further, the requirement of a P'_2 residue in these compounds was underscored by the observation that neither *N*-(cyanoacetyl)-L-phenylalanine nor (*R*)-2-benzyl-4-oxo-5-cyanopentanoic acid, which are effective mechanism-based inactivators of CPA,[6] inactivate NEP.

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Table I. Kinetic Parameters for the Inactivation of NEP and ACE by Compounds 1 and 2

	NEP		ACE[a]	
kinetic constants	1	2	1	2
k_{inact} (s^{-1})	0.071	0.022		0.08
K_m (mM)	5.1	2.38		10.5
K_i (μM)	14	17	>10000	65
k_{cat}/k_{inact}	1340	4700		8300

[a] Compound 1 is not a mechanism-based inactivator for ACE (ref 7).

Compounds 1 and 2 inactivated NEP in a time-dependent manner. Enzyme inactivation showed saturation and followed pseudo-first-order kinetics. Double-reciprocal plots of the first-order inactivation rate constant (k_{obs}) versus inactivator concentration were linear for both compounds and the kinetic parameters k_{inact} and K_m were evaluated to be 0.071 min^{-1} and 5.1 mM, and 0.022 min^{-1} and 2.38 mM for 1 and 2, respectively. The inactivation of NEP was indicative of covalent modification of the active site. Exhaustive dialysis of NEP, which had been completely inactivated by compounds 1 or 2, regenerated only 13% and 31% of the original enzymatic activity, respectively.

Protection from inactivation by compounds 1 and 2 was afforded in the presence of 10 μM thiorphan, a potent competitive inhibitor of NEP.[11] Furthermore, treatment of compounds 1 and 2 as inhibitors according to the method of Dixon[12] for the NEP-catalyzed hydrolysis of the peptide substrate dansyl-D-Ala-Gly-pNO_2-Phe-Gly[13] (DAGNPG) revealed a competitive mode of inhibition. The reversible inhibition constants (K_i) were measured at 14 and 17 μM for 1 and 2, respectively. These results strongly suggested that these molecules have a high affinity for the enzyme and that the inactivation is active site directed.

Partition ratios (k_{cat}/k_{inact}), evaluated by the titration method,[14] were measured to be 1340 and 4700 for 1 and 2, respectively. On the basis of these values, the kinetic parameter k_{cat} for 1 and 2 was estimated to be 95 and 103 min^{-1}, respectively. The higher partition ratio noted for compound 2 compared to its ketomethylene analogue 1 is consistent with our earlier studies with CPA.[6] L-Phenylalanyl-L-phenylalanine was isolated as the product of hydrolysis of compound 2.[15] However, attempts to identify the turnover product from compound 1 have thus far been unsuccessful. It is pertinent to note that metabolically essential enzymes such as NEP may require inactivators that down regulate the biological action of the target enzyme but do not completely abolish enzymic activity. Compounds 1 and 2, and other such molecules having similar partition ratio profiles as those reported above, potentially fulfill this expectation.

Table I presents the results of the kinetic analysis of the inactivation reaction of NEP using compounds 1 and 2 with those reported in our earlier investigation of ACE.[7] The kinetic parameters for the inactivation and turnover of ACE and NEP by compound 2 were quite similar, but the inactivation of NEP was not fully irreversible as was the case with ACE. The ketomethylene analogue 1 inactivated NEP, and its K_i value was indicative of a reasonably high affinity for NEP. In contrast, compound 1 was not a mechanism-based inactivator of ACE and bound only weakly to the enzyme as a reversible inhibitor.[7] Previous studies had also identified the importance of the NH group at the corresponding position in *N*-carboxymethyl dipeptide inhibitors[16] and peptide substrates[17] of ACE. The competitive inhibition and inactivation experiments reported in the present study reveal that the presence of a penultimate amide bound in compound 2 is not a critical requirement for reversible binding to the NEP active site and that the replacement of the NH of the peptidic compound 2 by a CH_2 group (compound 1) is tolerated by the enzyme for the chemistry of inactivation. This feature thus provides a motif for the design of mechanism-based inactivators which selectively differentiate between NEP and ACE.

Table II. Comparative Study on the Inactivation of Other Related Zinc Proteases with Compounds 1 and 2

	compound 1[a]		compound 2[a]		
		turnover products		turnover products	
enzyme	inactivation	Phe	inactivation	Phe	Phe-Phe
leucine aminopeptidase	no	no	no	no	no
carboxypeptidase A	no	yes	yes[b]	yes	no
carboxypeptidase B	no	yes	no	yes	no
thermolysin	no	no	no	yes	no
collagenase	no	no	no	yes	no

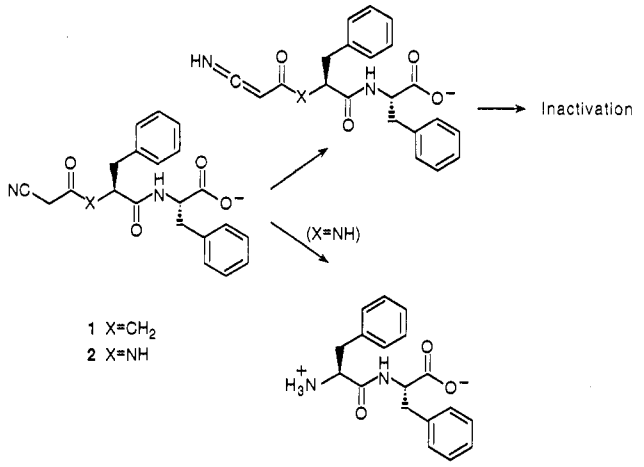
[a] The proteases were incubated with 10 mM concentrations of compound 1 or 2, and their enzymatic activity was monitored periodically. [b] Slow inactivation of CPA was observed due to the formation of *N*-(cyanoacetyl)-L-phenylalanine[6] from the CPA-catalyzed hydrolysis of compound 2.

The results of an inactivation study of five other zinc proteases with compounds 1 and 2 are summarized in Table II. None of these proteases were inactivated by the ketomethylene analogue 1. A slow inactivation of CPA was observed with the peptidic molecule 2 after the compound was hydrolyzed to form the CPA inactivator, *N*-(cyanoacetyl)-L-phenylalanine. However, the compound was ineffective toward carboxypeptidase B, thermolysin, collagenase, and leucine aminopeptidase. L-Phenylalanine was identified as the product of hydrolysis of compounds 1 and 2 with a number of the enzymes, but phenylanalyl-L-phenylalanine was absent as a product in the reaction mixtures. These observations provide further evidence for the specificity of compound 1 for NEP. The selectivity exhibited by compound 2 for NEP and ACE may have an important pharmacological consideration for the development of dual-action antihypertensive agents, since such compounds may be used to simultaneously inhibit degradation of ANF and angiotensin I.

The less stringent structural requirements observed for binding at the active site of NEP are in agreement with previous substrate specificity and competitive inhibition studies. These studies have suggested that, whereas there is a close resemblance in the general topologies of the active sites of NEP and ACE, there are subtle differences in the subsite specificities of the two enzymes. Comparative studies with thiol[10] and carboxyalkyl[18] inhibitors have shown the greater flexibility of the NEP active site for accommodating structural variations such as the position of the zinc-chelating moiety, large hydrophobic P'_1 residues, and retroinversion of the ultimate amide bond,[19] without adversely affecting binding affinities.

On the basis of the earlier studies on CPA,[6] ACE,[7] renal dipeptidase,[20] and other examples describing nitrile arrangements for enzyme inactivation,[21] we propose that NEP inactivation by compounds 1 and 2 involves an enzymic deprotonation, initiated either by promoted-water attack or by an active-site basic residue, followed by rearrangement to a ketenimine (Scheme I). This reactive

Scheme I



intermediate then traps an active-site nucleophile to inactivate the enzyme.

Experimental Section

The syntheses of compounds 1 and 2 have been previously reported.[7] Kinetic studies were performed on a Gilford Fluoro IV Fluorometer and on a Perkin-Elmer Lambda 3B UV/vis spectrometer. Recombinant human NEP isolated from mammalian CHO cell lines was provided by Dr. R. H. Bridenbaugh from Genentech. The enzyme was greater than 99% pure as judged by SDS–polyacrylamide gel electrophoresis. The fluorogenic NEP substrate DAGNPG was purchased from Calbiochem and was also synthesized as previously described.[13] Carboxypeptidases A and B, thermolysin, collagenase (type VII), and leucine aminopeptidase (type III-CP, from porcine kidney) were purchased from Sigma. D,L-Thiorpan and the enzyme substrates L-leucinamide, *N*-[3-(2-furyl)acrolyl]glycyl-L-leucine amide, *N*-[3-(2-furyl)acrolyl]-L-leucylglycyl-L-prolyl-L-alanine, and *N*-hippuryl-L-arginine were also from Sigma. The CPA substrate *O*-(*trans*-*p*-chlorocinnamoyl)-L-3-phenyllactic acid was synthesized according to the procedure of Ghosh et al.[6] Analytical HPLC analysis of the compounds were performed on a C-18 reverse-phase column (Altex, 0.46 × 25 cm) using a 1 mL/min flow rate and a 20–75% linear gradient of acetonitrile in 0.1% aqueous TFA over 25 min. Compounds were detected at 214 or 254 nm.

Enzyme Assay. The continuous fluorometric assay procedure of Florentin et al.[13] was used for monitoring the NEP-catalyzed hydrolysis of DAGNPG at 37 °C. Typically, the NEP assay was carried out in thermostated cuvettes containing 1 mL of 100 mM DAGNPG in 50 mM Tris, pH 7.4 (assay solution), and enzyme activity was measured by monitoring fluorescence increase as a function of time (λ_{ex} 342 nm, λ_{em} 562 nm).

CPA and CPB activities were measured by monitoring the enzymatic hydrolysis of *O*-(*trans*-*p*-chlorocinnamoyl)-L-3-phenyllactic acid[22] and *N*-hippuryl-L-arginine,[23] respectively. Collagenase and thermolysin were spectrophotometrically assayed with N-[3-(2-furyl)acrolyl]glycyl-L-leucine amide[24] and *N*-[3-(2-furyl)acrolyl]-L-leucylglycyl-L-prolyl-L-alanine,[25] respectively. Leucine aminopeptidase activity was measured by following the enzymatic hydrolysis of L-leucine amide.[26]

Kinetic Studies. The inactivation experiments and kinetic analyses were carried out according to the method of Ghosh et al.[6] Stock solutions of 1 and 2 were prepared in *p*-dioxane. Inactivation reactions were carried out at 25 °C in a total volume of 100 μL. The reaction mixture contained 0.5 μM NEP in 50 mM HEPES, pH 7.4, final concentrations of 1 or 2 of 0.5–7.5 μM, and 10% *p*-dioxane. At regular intervals, 10-μL aliquots were withdrawn and diluted into 990 μL of assay solution, and the enzyme activity was monitored immediately.

Competition experiments were performed in the presence of the reversible inhibitor, thiorphan.[11] A 1 μM solution of NEP was preincubated at 25 °C for 5 min with 10 μM thiorphan in 50 mM HEPES, pH 7.4. Subsequently, compound 1 or 2 was added to give final concentrations of 5 mM inactivator and 10% *p*-dioxane, and the enzymatic activity was monitored periodically. Control experiments were performed under similar conditions with NEP and thiorphan in the absence of the inactivators and by incubating NEP with compounds 1 and 2, respectively.

Compounds 1 and 2 were studied as reversible inhibitors of NEP at concentrations well below their respective K_m values using two different concentrations of the substrate DAGNPG (100 and 500 μM). A series of assay mixtures containing both the substrate and various inactivator concentrations (1–50 μM) were prepared in 50 mM HEPES, pH 7.4, supplemented with 5% *p*-dioxane. NEP was added to afford a final concentration of 0.5 μM in a total volume of 1 mL. The enzymatic activity was measured immediately.

The partition ratios were determined as follows: a series of solutions (30 μL final volume), each containing 0.5 μM NEP and various molar equivalents of inhibitors were prepared in 50 mM HEPES, pH 7.4, to give inactivator/NEP ratios ranging from 1 to 2600 and 1 to 6000 for 1 and 2, respectively. The solutions were stirred gently at 4 °C for 16 h. Subsequently, a 10-μL aliquot of each mixture was added to 990 μL of assay solution and the activity was measured immediately. Control experiments were carried out in the absence of the inactivators.

Product analysis was carried out by incubating NEP (0.5 μM) in the presence of 10 mM solutions of 1 or 2 in 50 mM HEPES, pH 7.4 supplemented with 10% *p*-dioxane at room temperature for 150–210 min until no more enzymic activity could be detected. Subsequently, the solutions were filtered through Centricon-30 devices (pretreated with 1% bovine serum albumin to saturate nonspecific protein binding sites on the membrane) at 5000*g* for 1 h and the filtrate was subjected to reverse-phase HPLC analysis. The retenate containing inactivated NEP was reconstituted to a volume of 100 μL in the same buffer, and the process of concentration and reconstitution was repeated twice. The residual enzyme activity of the recovered enzyme was subsequently measured.

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