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Substitution of potential metal-coordinating amino acid residues in the zinc-binding site of endopeptidase-24.11

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Neutral endopeptidase (EC 3.4.24.11; NEP) is a membrane-bound zinc-metallopeptidase. The catalytic zinc ion is coordinated to three amino acid residues (His^{538}, His^{587} and Glu^{646}) and a water molecule. Here, we have systematically substituted potential metal-coordinating amino acid residues (His, Glu, Asp, Cys, Tyr, Ser) for each of the three zinc ligands of NEP using a recombinant polymerase chain reaction procedure. NEP mutants at positions 583 and 587 were devoid of catalytic activity. However, Glu^{587} NEP and Cys^{583} NEP were able to bind partially a tritiated inhibitor, the binding of which is dependent on the presence of the zinc atom. At position 646, the aspartate and cysteine mutants exhibited activity. For both mutants K_m values were unaltered but k_{cat} values were decreased by about 20-fold. Both mutants bound the tritiated inhibitor with K_d values similar to that of the wild-type enzyme. Our data suggest that neither histidine-583 nor -587 can be replaced by any other ligands. On the other hand, the glutamic acid at position 646 can be converted to an aspartic acid or a cysteine indicating the importance of a negative charge at this position.

Neutral endopeptidase-24.11; Zinc-binding site; Catalytic mechanism

1. INTRODUCTION

The zinc-dependent hydrolase neutral endopeptidase-24.11 (EC 3.4.24.11, NEP; neprilysin, CD 10, CALLA) is a mammalian plasma membrane ectoenzyme [1]. NEP is distributed in many tissues including kidney and intestinal microvilli, the central nervous system, and the immune system. NEP is involved in the extracellular catabolism of biologically active peptides. According to their antinociceptive and antihypertensive properties, NEP inhibitors are of great interest [2].

NEP is a member of a zinc-metalloprotease family comprising among others the bacterial thermolysin (EC 3.4.24.4, TLN) which is the best characterized [3]. They all contain in their active site the conserved motif HExxH in which the Glu residue participates as a general base in the catalytic mechanism and the two His residues coordinate the zinc atom [4]. Among the members of this family NEP seems to be the most related to TLN; indeed both enzymes cleave peptide bonds on the amino side of hydrophobic residues. Moreover, NEP and TLN are equally sensitive to inhibition by phosphoramidon [5] and the NEP inhibitors thiorphan and retro-thiorphan which inhibit both enzymes with the same stereochemical dependence have been co-crystallized with TLN [6,7]. Finally, despite little overall homology between the primary structures of both enzymes [8] most of the already characterized amino acid residues located in the active site of NEP have their counterpart in TLN. Indeed, in addition to the amino acid residues of the common HExxH sequence, Glu^{646} which has been shown to be the third zinc ligand of NEP is equivalent to Glu^{166} in TLN [9]. Similarly, His^{711} of NEP involved in the stabilization of the tetrahedral intermediate during the transition state is analogous to His^{231} in TLN [10,11]. Finally, Val^{580} and Arg^{747} of NEP involved in substrate binding are equivalent in TLN to Val^{139} and Arg^{203}, respectively [12,13].

Zinc ions can take on a regulatory, structural, or catalytic role in enzymes. X-ray diffraction studies have allowed the characterization of structural and catalytic zinc-binding sites. In all analyzed enzymes a structural zinc atom is coordinated to four cysteine residues, whereas a catalytic zinc atom is coordinated to three amino acid residues and an activated water molecule [14,15]. In both cases the coordination is that of a distorted tetrahedron. The major zinc-binding amino acid side chains for catalytic zinc ions are imidazole (His), carboxyl (Asp, Glu) and thiol (Cys). In the present study we assess the performance of potential metal-coordinating residues (His, Asp, Glu, Cys, Ser and Tyr)

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Abbreviations B_{max}, maximum binding; NEP, neutral endopeptidase-24.11, CALLA, common acute lymphoblastic leukemia antigen; octylglucoside, *N*-octyl-β-D-glucopyranoside; HACBO-Gly, *N*-[(2*RS*)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]-glycine; MES, 2-(morpholino)ethanesulfonic acid; TBS, Tris-buffered saline

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[16] in the context of the zinc-binding site of NEP. Our biochemical data provides further information on zinc-binding in a structural and functional perspective.

2. EXPERIMENTAL

2.1. *Vector construction and site-directed mutagenesis*

A 3,868 bp DNA fragment (*Hin*dIII–*Bam*HI) from the already described expression vector pSVENK19 [17] was introduced in M13mp19 RF DNA. Using site-directed mutagenesis on single-stranded DNA [18] the sequence CTGCAG recognized by *Pst*I was changed to CTGCAT. This silent mutation did not modify the amino acid sequence of NEP. A 3,100 bp DNA fragment (*Bst*EII–*Bam*HI) containing the mutated region was then reintroduced in pSVENK19 to generate pSVENK20. These manipulations will allow us to use a 663 bp DNA fragment (*Pst*I–*Bst*EII) as a cassette for mutagenesis using PCR procedures.

Mutagenesis by PCR was performed according to the procedure described in [19] using the pfu Taq polymerase (Stratagene, Palo Alto). The PCR product was purified from agarose gel, then precipitated in 95% ethanol containing 2% potassium acetate and digested by *Pst*I and *Bst*EII. The 663 bp DNA fragment (*Pst*I–*Bst*EII) containing the mutated region was substituted for the equivalent non-mutated fragment in pSVENK20. The presence of the mutations and the absence of non-specific mutations in the expression vectors were confirmed by sequencing the 663 bp mutated region.

2.2. *Transfection of COS-1 cells, binding of [^{125}I]2B12 monoclonal antibody and [^{3}H]HACBO-Gly to COS-1 cells*

Wild-type and mutated enzymes were obtained by transient expression in COS-1 cells as previously described in [9]. The binding of [^{125}I]2B12 monoclonal antibody [20] was performed on intact transfected cells as described previously [21], and was used to monitor cell surface expression of NEP. The radiolabelled inhibitor [^{3}H]HACBO-Gly (30 Ci/mmol) [22] was synthesized in the laboratory of B.P. Roques (Paris) and tritiated at the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France). Inhibitor binding was performed as described in [9] except that cells were dissolved in Solvable (New-England Nuclear) and radioactivity was measured using Formula-989 (New-England Nuclear) B_{max} values were determined after quantitation of soluble extracts by immunoblotting.

2.3. *Enzyme assays and immunoblotting*

NEP was solubilized in TBS containing 1% octylglucoside [9]. The activity of NEP in the extracts was measured using the tritiated substrate (tyrosyl-[3,5-^{3}H])(D-Ala$_2$)-Leu-enkephalin (50 Ci/mmol) (Research Product International Inc., Mount Prospect, IL) as previously described [9] except that incubations were carried out in 50 mM MES, pH 6.5, at 37°C. All enzymatic assays were performed under initial velocity conditions. The concentration of wild-type or mutated enzymes was determined by immunoblotting using purified rabbit kidney NEP as a standard as described in [9] except that the bands were visualized using the ECL System from Amersham. The sensitivity of the non-mutated and mutated enzymes towards HACBO-Gly and 1,10-phenanthroline were determined from dose–response curves. In the case of competitive inhibition, K_i values corresponded to the IC_{50} values, since the concentration of substrate (50 nM) was well below the K_m value.

3. RESULTS

3.1. *Expression of recombinant enzymes*

Wild-type and mutant plasmids were transiently expressed in COS-1 cells. All the recombinant proteins were recognized at the cell surface by the [^{125}I]2B12 monoclonal antibody (data not shown). Moreover, immunoblot analysis of cell extracts showed that the mutant enzymes had molecular weights very similar to that of the non-mutated recombinant enzyme (94 kDa) (data not shown). These results indicate that the mutant enzymes are expressed at the cell surface as efficiently as the non-mutated enzyme and are fully glycosylated.

3.2. *Mutations of His^{583} or His^{587}*

Both histidine residues belong to the canonic sequence HExxH. Substitutions at positions 583 or 587 had dramatic effects on enzymatic activity: none of the mutants had significant enzymatic activity in the conditions of the assay (Table I). This lack of activity might be the consequence of a decreased affinity for the zinc ion. To test this hypothesis, $ZnCl_2$ at concentrations ranging from 10^{-8} to 10^{-3} M was added in the incubation buffer. The addition of $ZnCl_2$ did not reactivate mutant enzymes. Binding experiments using 10 nM [^{3}H]HACBO-Gly were also performed on the mutant enzymes in the absence or in the presence of added $ZnCl_2$ to test for the presence of the zinc ion in the active site. Indeed, when it is used in a nanomolar range of concentrations this potent inhibitor of NEP is strictly dependent on the presence of a well-positioned zinc ion in the active site of the enzyme for binding [22,23]. In absence of exogenous zinc Glu^{587} NEP was the only mutant able to significantly bind the tritiated inhibitor. As shown in Fig. 1, binding was enhanced by addition of $ZnCl_2$ up to 10^{-6} M. Higher zinc concentrations led to a decrease in inhibitor binding which was also ob-

Table I

Properties of mutated and non-mutated enzymes

Enzymes	Enzymatic activity	Inhibitor binding
His^{583} NEP	+	+
Cys^{583} NEP	–	+[a]
Asp^{583} NEP	–	–
Glu^{583} NEP	–	–
Tyr^{583} NEP	–	–
His^{587} NEP	+	+
Cys^{587} NEP	–	–
Asp^{587} NEP	–	–
Glu^{587} NEP	–	+
Tyr^{587} NEP	–	–
Glu^{646} NEP	+	+
Asp^{646} NEP	+	+
Cys^{646} NEP	+	+
His^{646} NEP	–	–
Tyr^{646} NEP	–	–
Ser^{646} NEP	–	–

Enzymatic activity was determined with the tritiated substrate (D-Ala$_2$)Leu-enkephalin and inhibitor binding experiments were performed with the tritiated competitive inhibitor [^{3}H]HACBO-Gly.

[a] This mutant bound the tritiated inhibitor only in presence of exogenous zinc

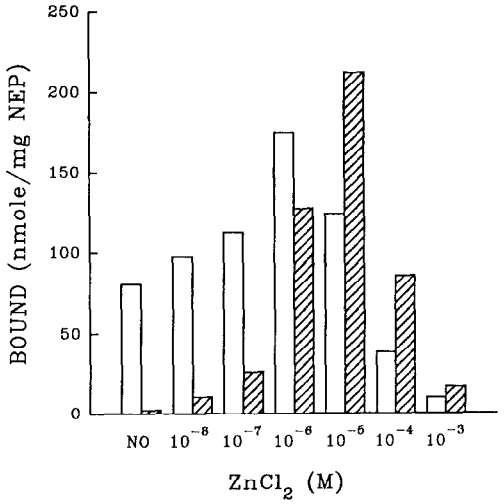

Fig. 1. Binding of [^{3}H]HACBO-Gly to Glu^{587} and Cys^{583} NEPs. Binding was performed as described in section 2 using 10 nM of the tritiated inhibitor. Increasing amounts of $ZnCl_2$ were added to the incubation medium. Open bars = Glu^{587} NEP; hatched bars = Cys^{583} NEP

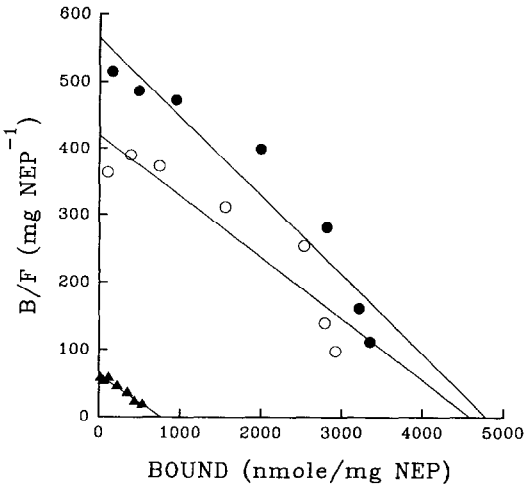

Fig. 2. Scatchard plots of [^{3}H]HACBO-Gly binding to non-mutated and mutated NEPs. Binding was performed as described in section 2. K_d and B_{max} values (see text) were deduced from the slopes and are the mean of three independent experiments. Filled circles = non-mutated NEP; open circles = Asp^{646} NEP; filled triangles = Cys^{646} NEP.

served for wild-type NEP (data not shown). Maximum binding for Glu^{587} NEP was only 6% of the binding observed with the non-mutated enzyme. Cys^{583} NEP was also able to bind the tritiated inhibitor but only in the presence of additional zinc and showed a maximal binding at 10^{-5} M $ZnCl_2$ (Fig. 1). In these conditions this binding represented 7.5% of the wild-type binding. These results are consistent with a decrease in affinity of these two mutated proteins for the zinc ion. However, the absence of saturation for inhibitor binding did not allow us to determine significant binding parameters.

3.3. *Mutations of Glu^{646}*

Mutations affecting Glu^{646} of NEP are less drastic with regard to the catalytic activity. Indeed, in addition to Asp^{646} NEP which was characterized previously [9], Cys^{646} NEP showed a substantial activity in absence of exogenous zinc (Table I). Addition of zinc up to a concentration of 10^{-6} M did not enhance activity of the mutant enzymes, but higher concentrations of $ZnCl_2$ decreased enzymatic activity as observed for the wild-type enzyme (results not shown). This inhibition of enzymatic activity already observed for the renal enzyme [24] might be attributed to the presence of a putative inhibitory zinc-binding site as proposed for carboxypeptidase A [25]. Kinetic parameters were measured for Asp^{646} NEP and Cys^{646} NEP. Table II shows that the 20-fold decrease in specific activity (k_{cat}/K_m) for both mutants is fully accounted for a decrease in k_{cat}. That the K_m values for both mutants are very similar to that of the non-mutated enzyme suggest that the structure of the active site has not been grossly altered by these mutations. This hypothesis is supported by a similar sensitivity of the mutant and non-mutated enzymes to HACBO-Gly (Table II). However, both mutant enzymes exhibited a higher susceptibility to inhibition by the chelating agent 1,10-phenanthroline than the non-mutated enzyme (Table II), suggesting a slightly reduced affinity of both mutants for the zinc ion. To confirm the presence of a well-positioned zinc ion in the active site of Asp^{646} NEP and Cys^{646} NEP, we performed binding experiments using [^{3}H]HACBO-Gly in absence of exogenous zinc. Binding parameters were determined from Scatchard plots (Fig. 2). K_d values

Table II

Kinetic parameters of mutated and non-mutated enzymes

Enzymes	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m min$^{-1}\cdot\mu$M^{-1})	K_i[a] (nM)	IC_{50}[b] (μM)
Wild-type NEP	44 ± 5	700 ± 35	16	3 ± 1	89 ± 8
Asp^{646} NEP	42 ± 5	36 ± 10	0.85	8 ± 2	19 ± 4
Cys^{646} NEP	50 ± 6	40 ± 2	0.80	5 ± 1	14 ± 1

K_m and k_{cat} values are the mean of three determinations performed as described in section 2.5.

[a] K_i values are for the inhibitor HACBO-Gly.
[b] IC_{50} values are for 1,10-phenanthroline

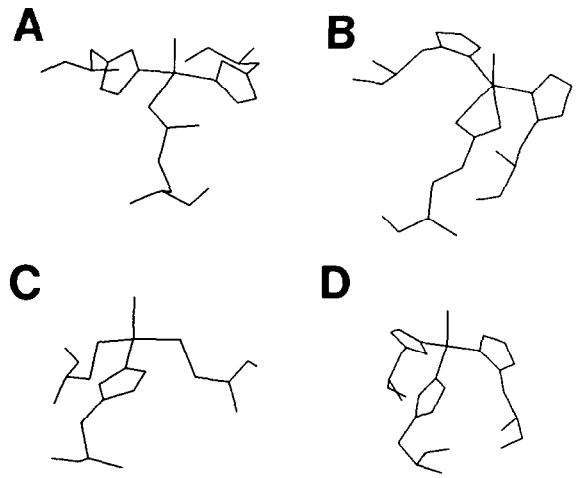

Fig. 3. Zinc-binding sites of several zinc-enzymes (A) Thermolysin (PDB 3TLN entry), zinc being coordinated to two histidine residues, a glutamic acid residue and a water molecule [3]. (B) Carboxypeptidase A (PDB 5CPA entry), zinc being coordinated to two histidine residues, a glutamic acid residue and a water molecule [30]. (C) Alcohol dehydrogenase (PDB 8ADH entry), zinc being coordinated to two cysteine residues, a histidine residue and a water molecule [31]. (D) Carbonic anhydrase (PDB 2CAB entry), zinc being coordinated to three histidine residues and a water molecule [29]

were found to be 10 ± 1.5 nM and 12 ± 1 nM for Asp^{646} NEP and Cys^{646} NEP, respectively. These values are similar to that determined for the non-mutated enzyme (8 ± 1 nM). On the other hand, B_{max} values were very different for both mutants since they were estimated to be 4,270 ± 440 nmol/mg NEP and 755 ± 45 nmol/mg NEP for Asp^{646} NEP and Cys^{646} NEP, respectively. B_{max} value for Cys^{646} NEP was 5 times lower than that of the non-mutated enzyme (4,600 ± 350 nmol/mg NEP) indicating that only a portion of the molecules bound the tritiated inhibitor. Addition of zinc up to 10^{-6} M did not enhance [^{3}H]HACBO-Gly binding for wild-type enzyme nor for the mutant enzymes, but higher zinc concentrations decreased this binding (results not shown).

4. DISCUSSION

X-ray diffraction studies of zinc-metallopeptidases and other zinc-enzymes have shown that different amino acid side chains can act as ligands of catalytic zinc ions (Fig. 3) [26]. In the present study, we have evaluated the possibility that other amino acid side chains could replace the three zinc ligands of NEP. The choice of the amino acid residues in the substituted positions was dictated by their presence in the metal-binding site of other zinc-enzymes.

All substitutions performed at positions 583 and 587 resulted in inactive enzymes. However, two of the mutant enzymes, Cys^{583} NEP and Glu^{587} NEP, bound partially [^{3}H]HACBO-Gly which is dependent on the presence of zinc in the active site for binding. Given the small amounts of recombinant enzymes it has not been possible to verify the presence of the zinc ion by direct chemical or isotopic methods. The lack of [^{3}H]HACBO-Gly binding observed for other mutant enzymes does not necessarily mean that zinc ion is absent from the active site. Indeed, zinc binding with a very low affinity or in a position preventing interaction with the hydroxamate group of the inhibitor may also result in the absence of [^{3}H]HACBO-Gly binding. That Cys^{583} NEP and Glu^{587} NEP are inactive despite their ability to bind partially the zinc ion can be explained by several non-exclusive hypotheses: (1) mutations introduced a distortion in the putative α-helix containing the motif HExxH and consequently a small change in the spatial position of the neighbouring Glu^{584} which is essential for catalytic activity [17]; (2) Glu^{587} and Cys^{583} side chains which can simultaneously coordinate the zinc atom and accept hydrogen bonds [27] form a hydrogen bond with the zinc-coordinated water molecule, thus changing its spatial location and consequently preventing nucleophilic attack of the substrate; (3) the decrease in affinity for zinc could reflect a small change in the spatial position of the zinc ion itself that could prevent polarization of the carbonyl group of the substrate that is the fifth zinc ligand during catalysis [3]. Thus, substitutions at positions 583 and 587 suggest that histidine residues at these two positions are strictly required for the activity of the enzyme.

The situation is somehow different at position 646 since both Asp^{646} NEP and Cys^{646} NEP exhibited some activity. K_m values for the substrate and K_d values for [^{3}H]HACBO-Gly are similar to that of the non-mutated enzyme indicating that these mutations do not induce a gross alteration of the active site of these enzymes. However, the slower rate of substrate hydrolysis and the higher sensitivity to the chelating agent 1,10-phenanthroline observed for both mutants can be explained by a small displacement of the zinc ion resulting from the mutations, as discussed previously [9]. The 5-fold decrease in B_{max} for Cys^{646} NEP in comparison with both Asp^{646} NEP and the wild-type enzyme suggests heterogeneity of the Cys^{646} mutant enzymes. Indeed, only 20% of Cys^{646} NEP molecules were able to bind the tritiated inhibitor. The remaining molecules could be structurally altered by the formation of a non-native disulfide bridge involving Cys^{646}. Thus, if one takes into account the proportion of Cys^{646} NEP able to bind the tritiated inhibitor, the k_{cat}/K_m value should decrease 4-fold only rather than 20-fold. This observation indicates that cysteine is a better ligand for zinc than aspartic acid at position 646 of NEP. Furthermore, it confirms the greater ability for thiolate than carboxylate to coordinate the zinc ion [28]. It is important to note that a negatively charged ligand is necessary at position 646 for zinc-coordination. Indeed, both aspartate and glutamate are negatively charged residues and the thiol side chain of cysteine is also negatively charged as it complexes a metal ion in a protein [27]. The nature of the

zinc ligands in His 646 NEP is identical to that found in carbonic anhydrase (CA) (Fig. 3D). However, this NEP mutant was both inactive and unable to bind the tritiated inhibitor suggesting that Glu^{646} of NEP cannot be replaced by a histidine residue. This observation can best be explained by the fact that the three zinc ligands are not in the same structural context in NEP and CA. Indeed, the three zinc ligands of TLN and probably of NEP are on two adjacent α-helices, whereas the three equivalent residues of CA are on two adjacent β-strands [29]. However, the zinc-binding site of NEP and CA share common features since they are characterized by a short spacing between the first two ligands, a longer spacing between the second and the third ligand, and probably a distorted tetrahedral geometry [14].

Taken together, our results suggest that in the context of NEP a histidine ligand can neither be replaced efficiently by another potential ligand nor replace the Glu residue as ligand. This is in good agreement with the unique structural features of the histidine side chain that is characterized by its conformational rigidity and the possibility of hydrogen bonding for the nitrogen atom of the imidazole ring not involved in zinc-binding [27]. On the other hand, that an aspartic or a cysteine can replace a glutamic acid can be explained by the relative similarity of their side chain.

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