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Exploration of the catalytic site of endopeptidase 24.11 by site-directed mutagenesis

Histidine residues 583 and 587 are essential for catalysis

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Direct comparison of the primary structure of neutral endopeptidase (NEP, EC 3.4.24.11) with that of thermolysin, a bacterial metalloendopeptidase with a similar specificity, has revealed very few similarities between the two sequences, except for two conserved short segments. In thermolysin, these segments contain several of the residues involved in catalysis, including two zinc coordinating histidines (His-142 and His-146) and a third histidine (His-231) involved in stabilizing the transition state through hydrogen bonding. The role of the corresponding histidines in NEP (His-583, His-587 and His-637) was explored by site-directed mutagenesis of NEP cDNA and expression of the mutated cDNA in COS-1 cells. Substitution of either His-583 or His-587 of NEP for Phe completely abolished the activity and Zn-directed inhibitor recognition of the recombinant enzyme, suggesting that these residues play a role similar to His-142 and His-146 of thermolysin as zinc ligands. In contrast, substitution of His-637 for a phenylalanine residue was without effect on enzyme activity.

Endopeptidase 24.11; Histidine; Catalytic site; Site-directed mutagenesis

1. INTRODUCTION

Neutral endopeptidase (NEP, EC 3.4.24.11) is a transmembrane zinc-metalloendopeptidase found in the central nervous system and in many peripheral tissues [1]. The role of NEP is not yet fully established. However its preference for small peptides rather than proteins and its presence at the cell surface suggest a possible role in the inactivation of biologically active peptides [2]. There is evidence in favor of a role for NEP in the degradation of neuropeptides in the central nervous system [3,4]. Furthermore, inhibitors of NEP such as thiorphan and retrothiorphan partially protect the degradation of endogenous enkephalins and induce naloxone-reversible analgesic responses [5,6].

NEP shares several structural features with thermolysin, a bacterial metalloendopeptidase. Both enzymes are zinc-metalloendopeptidase and are inhibited by phosphoramidon [7,8]. Their S_1' subsite accepts exclusively hydrophobic amino acid residues [9], while a proline in the P_2' position decreases the interaction with both enzymes [10]. Furthermore an essential His residue present in the thermolysin active site was also found in NEP [11,12]. Finally, comparison of the primary structure of both enzymes has shown that many of the amino acids involved in catalysis and binding of the zinc atom in thermolysin are found in two protein segments that have been conserved in NEP [13,14]. We have recently used in vitro mutagenesis to test the involvement of these residues. Changing Glu-584 of NEP for either Val or Asp completely

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abolished the activity of the recombinant mutant enzyme thereby supporting the hypothesis that this Glu residue is important for NEP activity [15].

We have now studied the role of three other amino acids of NEP: His-583, His-587 and His-637 corresponding to His-142, His-146 and His-231 of thermolysin, respectively (see fig.1). In thermolysin, the two former histidines are ligands of the zinc atom while the latter appears to be involved in the stabilization of the transition state [16]. Our results suggest that His-583 and His-587 are most probably two of the three ligands of the zinc atom in NEP. However, substituting His-637 for Phe did not change the activity of NEP nor its affinity for inhibitors, indicating that His-637 is not directly involved in enzyme activity.

2. MATERIALS AND METHODS

2.1. *Materials*

Leu-enkephalin was from Institut Armand-Frappier (Laval, Qué.) and diethylpyrocarbonate from Sigma. The tritiated NEP inhibitor [^{3}H]-*N*-[(2*RS*)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]glycine ([^{3}H]HACBO-Gly, 30 Ci/mmol) [17] was synthesized in the laboratory of one of us (B.P.R.) and tritiated at the Commissariat à l'Energie Atomique (CEA, Saclay, France). All the other radiochemicals were from Amersham.

2.2. *Site-directed mutagenesis of NEP cDNA and expression in COS-1 cells*

Mutagenesis of NEP was performed by oligonucleotide-directed mutagenesis according to Taylor et al. [18] on a M13 subclone containing the proper fragment of NEP cDNA. Mutant cDNAs were screened by sequencing [19] and a fragment containing the mutated region was isolated from the replicating form of the M13 recombinant phage and substituted in pSVENK19, an expression vector using the SV40 early promotor to express NEP in COS-1 cells [15].

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

COS-1 cell suspensions were prepared in Tris-buffered saline (TBS) containing 4 mM EGTA. For both ^{125}I-2B12 Mab and [^{3}H]HACBO-Gly binding was assayed on 4×10^5 cells [15]. Cells were separated from unbound ligands by centrifugation in an Eppendorf centrifuge and the pellets washed twice with cold TBS. Radioactivity due to ^{125}I was determined directly on the cell pellets with a gamma counter whereas ^{3}H radioactivity was measured by liquid scintillation counting after dissolving the cells in Protosol (New-England Nuclear). The binding of ^{125}I-2B12 monoclonal antibody (Mab) was used to monitor the presence of NEP at the cell surface. This Mab recognizes a conformational epitope in the ectodomain of NEP (Aubry, LeGrimellec and Crine, unpublished). HACBO-Gly is a high-affinity non-hydrolysable NEP substrate-derived inhibitor [20].

2.4. *Purification of NEP and enzyme assays*

NEP was purified from COS-1 cells solubilized in TBS containing 1% *N*-octyl-β-D-glucopyranoside (octylglucoside) [21]. Immunoprecipitation of the enzyme was performed with 2B12 Mab as described elsewhere [22] and the amount of NEP was determined by a dot-blot assay [15]. Enzyme assays were performed with purified NEP in 0.05 M Tris-HCl, pH 7.4, at 25°C using [*tyrosyl*-3,5-^{3}H]Leu-enkephalin (25 nM; 34 Ci/nmol, Amersham) as a substrate. Separation of metabolites from the substrate was performed by chromatography on C_{18} Sep-Pak cartridges (Waters) as described elsewhere [22]. For K_m determinations, the concentration of the substrate was varied by the isotopic dilution method and the values were calculated from Eadie-Hofstee plots.

2.5. *Diethylpyrocarbonate modification of NEP*

Purified NEP was incubated in 0.05 M Tris-HCl, pH 7.4, for 45 min at 22°C in the presence or absence of 2 mM diethylpyrocarbonate (DEPC) [11]. In some experiments, 2 mM Phe-Leu was added before DEPC. Treatment was stopped by passage through a G-25 Sephadex column.

3. RESULTS

The pSVENK19 plasmid contains DNA sequences coding for rabbit kidney NEP inserted downstream of the SV40 early promotor. This vector has already been used to induce the expression of NEP after transfection in COS-1 cells. The recombinant NEP solubilized from COS-1 cell extracts is indistinguishable from NEP obtained from kidney brush borders according to several structural and catalytic criteria [15]. These observations allowed us to use the COS-1 cell expression system to test the involvement of histidines 583, 587, and 637 of NEP in catalysis by site-directed mutagenesis. In each instance, the histidine residue was changed for a phenylalanine residue. Expression vectors pSVENK19-3, pSVENK19-6 and pSVENK19-7, harboring mutations for His-583, His-587 and His-637, respectively, were thus obtained and used to transfect COS-1 cells. Part of the transfected cell culture was used to monitor the presence of enzyme at the cell surface by measuring the binding of ^{125}I-2B12 Mab to cell monolayers and the rest of the cell culture was lysed in the presence of octylglucoside. The recombinant enzyme present in the cell extract was immunoprecipitated with 2B12 Mab and quantitated by immunogold blots. The activity of equivalent immunoreactive amounts of either mutant or non-mutated recombinant NEPs was assayed by measuring the rate of [^{3}H]Leu-enkephalin hydrolysis.

No hydrolysis of the substrate above the background level was observed for either Phe-583-NEP or Phe-587-NEP after a 60 min incubation (fig.2a). Based on the sensitivity of our enzymatic assays, the specific activities of these mutated enzymes were therefore estimated to be at least 500-fold lower than that of the non-mutated recombinant enzyme. These results show that substituting a Phe residue for either His-583 or His-587 drastically reduces NEP activity.

To further probe the importance of these amino acid residues in the catalytic site of the enzyme, we next studied the binding of [^{3}H]HACBO-Gly to both the non-mutated and the mutated enzymes Phe-583-NEP and Phe-587-NEP. HACBO-Gly is a specific inhibitor of NEP [17] whose binding to the active site of the enzyme is strictly dependent on the presence of the Zn atom [20]. No binding was detected with cells expressing Phe-583 NEP or Phe-587 NEP (fig.3a). As binding was directly performed on suspensions of transfected cells these results could be explained by the failure of these mutated recombinant enzymes to reach the cell surface. In a control experiment, however, these cells were found to bind ^{125}I-Mab to the same extent as cells transfected with the non-mutated expression vector (not shown), suggesting that the normal amounts of mutated recombinant enzyme were reaching the cell surface. Moreover, mutations of amino acids present in the NEP active site but not involved in HACBO-Gly recognition, such as Glu-584, did not modify the affinity of the enzyme for the inhibitor [15].

It has been reported that an essential histidine residue is present at the active site of NEP [11]. This was shown by DEPC treatment of the native enzyme which resulted in virtually complete inac-

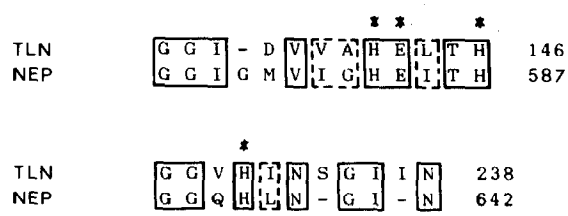

Fig.1. Homology between amino acid sequence of thermolysin (TLN) and NEP. Numbers refer to the last amino acid position in each protein segment. Asterisks indicate the position of Zn-coordinating and catalytic residues in thermolysin. Identical residues are boxed while conservative amino acid changes are indicated by broken lines. Gaps are repesented by dashes.

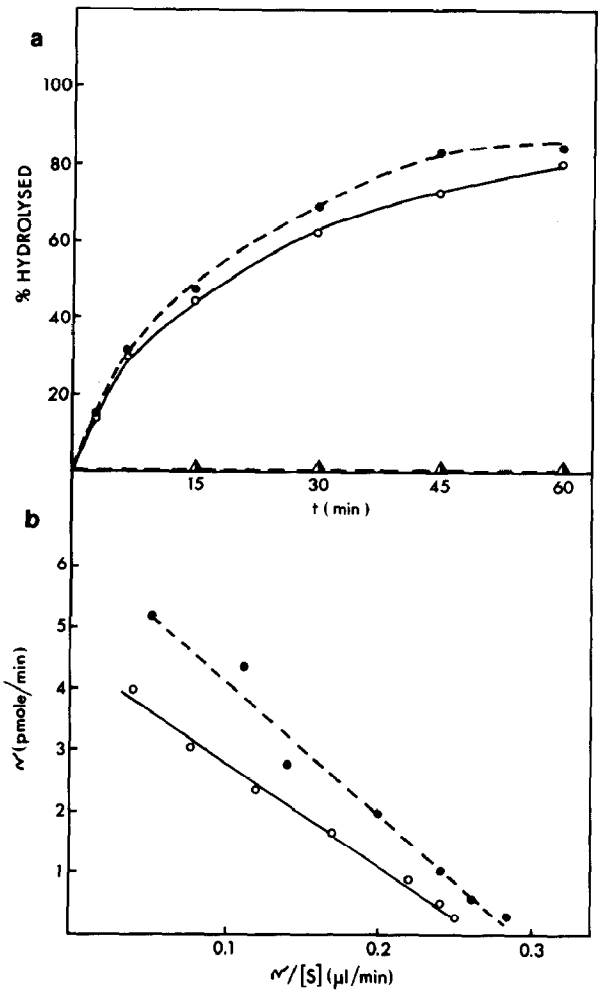

Fig.2. Activity of non-mutated and mutated NEP. COS-1 cells were transfected with either pSVENK19, pSVENK19-3, pSVENK19-6 or pSVENK19-7. The recombinant enzymes were purified from cell extracts by immunoprecipitation and assayed for [^{3}H]Leu-enkephalin hydrolysis. (a) Rate of hydrolysis of [^{3}H]Leu-enkephalin. The activity of non-mutated and mutated NEP was determined by measuring the rate of hydrolysis of [^{3}H]Leu-enkephalin (30 nM). (b) Eadie-Hofstee plots. (●) Non-mutated NEP; (○) Phe-637-NEP; (△) Phe-583 NEP; (▲) Phe-587-NEP. All assays were performed in triplicates.

tivation. In thermolysin, crystallographic data have shown the presence of His-231 at the active site [23]. According to Hangauer et al. [16], this histidine residue participates in the stabilization of both the Michaelis complex and the tetrahedral intermediate. As mentioned above, His-231 of thermolysin might correspond to His-637 in NEP (fig.1). To directly test the importance of His-637 in catalysis, we prepared a recombinant enzyme

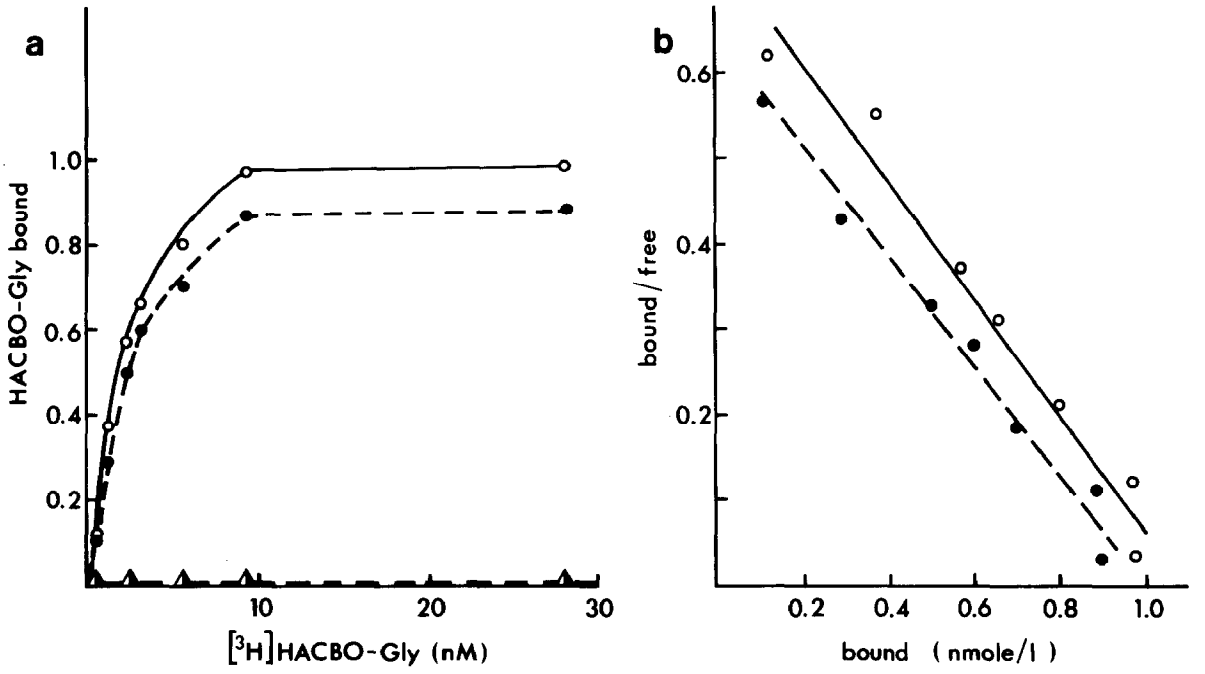

Fig.3. Binding of [^{3}H]HACBO-Gly to non-mutated and mutated NEP. Binding was performed on COS-1 cells transfected with either pSVENK19, pSVENK19-3, pSVENK19-6 or pSVENK19-7 as described in section 2. (a) Saturation curves of specific [^{3}H]HACBO-Gly binding to COS-1 cells: non-specific binding was determined in the presence of 1 μM thiorphan. All assays were performed in triplicates. (b) Scatchard plots: K_d values were deduced from the slopes and calculated from two independent experiments: non-mutated NEP; 1.4 nM, Phe-637-NEP: 1.6 nM. (●) Non-mutated NEP; (○) Phe-637-NEP; (△) Phe-583-NEP; (▲) Phe-587-NEP.

where this residue had been changed for a phenylalanine and we analyzed this mutant enzyme for catalytic activity, [^{3}H]HACBO-Gly binding and sensitivity to DEPC. Fig.2a shows that Phe-637-NEP hydrolysed the substrate at a rate similar to that of the non-mutated enzyme. The values of k_{cat} and K_m determined from Eadie-Hofstee plots were found to be very similar for both Phe-637-NEP (k_{cat} = 500 min^{-1}, K_m = 20 μM) and non-mutated NEP (k_{cat} = 550 min^{-1}, K_m = 22 μM) (fig.2b). Furthermore the affinity of Phe-637-NEP for [^{3}H]HACBO-Gly (K_d = 1.6 nM) was very similar to that of the non-mutated NEP (K_d = 1.4 nM) as shown by the Scatchard plots of fig.3b. Finally, both the mutated Phe-637-NEP and the non-mutated enzyme are inactivated to the same extent by DEPC treatment (table 1). In both cases this inactivation can largely be prevented by preincubating the enzyme in the presence of 2 mM Phe-Leu, a NEP competitive inhibitor (K_i = 20 μM). Taken together, these results strongly suggest that His-637 does not play any significant role in catalysis.

Table 1

Effects of DEPC modification of non-mutated and Phe-637-NEP

Enzyme	Enzyme activity		
	Control	2 mM DEPC	2 mM DEPC + 2 mM Phe-Leu
Non-mutated NEP	100%	15%	92%
Phe-NEP	100%	12%	95%

Enzyme activity corresponds to the initial rate of hydrolysis of 25 nM [^{3}H]Leu-enkephalin

4. DISCUSSION

The drastic reduction of the rate of substrate hydrolysis observed for the recombinant NEP mutants where His-583 or His-587 has been replaced by a phenylalanine residue suggests that these two histidines are essential for catalysis. Considering the similarity between this region of NEP and the sequence of thermolysin that contains the

two zinc-coordinating histidines, we propose that His-583 and His-587 of NEP are two of the zinc ligands. This conclusion is also supported by our observation that Phe-583-NEP and Phe-587-NEP are unable to bind HACBO-Gly, a non-hydrolysable substrate-derived inhibitor of NEP designed to interact with the metal atom [20].

Given the small amounts of recombinant enzyme synthesized by COS-1 cells, it has not been possible to directly verify the absence of a Zn atom in these mutant NEP molecules by chemical or isotopic methods. Therefore we cannot completely rule out the possibility that failure of these recombinant enzymes to hydrolyse the substrates at a significant rate and to bind HACBO-Gly is the result of a profound alteration of enzyme subsites. We however believe this to be a very unlikely possibility for two reasons: (i) other mutations introduced in the same area of the NEP molecule such as the substitution of Ile-585 for Phe had no effect on neither HACBO-Gly binding nor catalytic activity (Devault, unpublished); (ii) the non-conservative change of the active site Glu-584 for Val completely abolishes the catalytic activity but does not grossly distort the enzyme structure as indicated by the normal K_d for [^{3}H]HACBO-Gly [15].

In contrast the apparent lack of change in the catalytic and binding properties of NEP following replacement of His-637 by Phe, suggests that this His residue is not involved in the stabilization of the transition state and therefore does not correspond to His-231 of thermolysin. This is reinforced by the sensitivity of mutated Phe-637-NEP to chemical modification induced by the His reagent DEPC. Additional experiments are therefore required to assign all the amino acids present in the active site.

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