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Evidence that Asn^{542} of neprilysin (EC 3.4.24.11) is involved in binding of the P_2' residue of substrates and inhibitors

Natalie DION,* Hervé LE MOUAL,* Marie-Claude FOURNIÉ-ZALUSKI,† Bernard P. ROQUES,† Philippe CRINE* and Guy BOILEAU*‡

*Département de Biochimie, Faculté de Médecine, Université de Montréal, Montréal, Canada
and †Département de Chimie Organique, U 266 INSERM, UA 498 CNRS, 4, Avenue de l'Observatoire, 75006 Paris, France

Neprilysin (EC 3.4.24.11) is a Zn^{2+} metallopeptidase involved in the degradation of biologically active peptides, e.g. enkephalins and atrial natriuretic peptide. The substrate specificity and catalytic activity of neprilysin resemble those of thermolysin, a crystallized bacterial Zn^{2+} metalloprotease. Despite little overall homology between the primary structures of thermolysin and neprilysin, many of the amino acid residues involved in catalysis, as well as Zn^{2+} and substrate binding, are highly conserved. Most of the active-site residues of neprilysin have their homologues in thermolysin and have been characterized by site-directed mutagenesis. Furthermore, hydrophobic cluster analysis has revealed some other analogies between the neprilysin and thermolysin sequences [Benchetrit, Bissery, Mornon, Devault, Crine and Roques (1988) Biochemistry **27**, 592–596]. According to this analysis the role of Asn^{542} in the neprilysin active site is analogous to that of Asn^{112} of thermolysin, which is to bind the substrate. Site-directed mutagenesis was used to change Asn^{542} to Gly or Gln residues. The effect of these mutations on substrate catalysis and inhibitor binding was examined with a series of thiorphan-like compounds containing various degrees of methylation at the P_2' residue. For both mutated enzymes, determination of kinetic parameters with [D-Ala^2,Leu^5]enkephalin as substrate showed that the large decrease in activity was attributable to an increase in K_m (14–16-fold) whereas $k_{cat.}$ values were only slightly affected (2–3-fold decrease). This is in agreement with Asn^{542} being involved in substrate binding rather than directly in catalysis. Finally, the IC_{50} values for thiorphan and substituted thiorphans strongly suggest that Asn^{542} of neprilysin binds the substrate on the amino side of the P_2' residue by formation of a unique hydrogen bond.

INTRODUCTION

The Zn^{2+}-dependent peptidase neprilysin (EC 3.4.24.11; NEP; neutral endopeptidase; CD10; common acute lymphoblastic leukaemia antigen) is a 94 kDa mammalian ectoenzyme anchored in the plasma membrane with a type-II topology [1,2]. The cDNA sequences for the human, rabbit, rat and mouse enzymes have been elucidated and show a high degree of similarity [3–6]. It is also known that the slight variations in size of NEP between tissues is due to differences in the extent of glycosylation [7]. It is distributed in many tissues including kidney and intestinal microvilli, the central nervous system and the immune system. This enzyme is involved in the extracellular catabolism of biologically active peptides such as enkephalins and atrial natriuretic peptide. Because of their antinociceptive and antihypertensive properties, NEP inhibitors are of great interest (reviewed in [8]).

Of all the members of the Zn^{2+} metalloprotease/peptidase family [9,10], NEP seems to be related the most closely to thermolysin (EC 3.4.24.4; TLN), a bacterial enzyme whose three-dimensional structure has been determined by crystallography and X-ray diffraction [11]. In a recent classification of Zn^{2+} metalloprotease/peptidases based on the structure of the Zn^{2+}-binding site [12], both NEP and TLN were classified in the Gluzincin group. This group is characterized by the short HEXXH Zn^{2+}-binding consensus sequence and a glutamic acid residue as the third Zn^{2+} ligand. Both enzymes also cleave peptide bonds on the amino side of hydrophobic residues and are equally sensitive to inhibition by phosphoramidon, a natural TLN-specific inhibitor [13–15]. Moreover the well-known NEP thiol inhibitors, thiorphan [16] and retrothiorphan [17], inhibit TLN with apparently the same stereochemical dependence [18,19].

Despite little overall sequence homology, many amino acid residues located in the active site of TLN have their counterparts in NEP [4]. These include, in addition to the HEXXH consensus sequence found in many proteases of this family [20,21], Glu^{646} (Glu^{166} in TLN), which has been shown to be the third Zn^{2+} ligand of NEP [22], Asp^{650} (Asp^{170} in TLN), important for catalytic activity and probably involved in a triad with His^{583} and the Zn^{2+} ion [23], His^{711} (His^{231} of TLN), which is involved in the stabilization of the tetrahedral intermediate during the transition state [24,25], and Val^{580} and Arg^{747} (Val^{139} and Arg^{203} of TLN) which participate in substrate binding [26,27]. An apparent difference between NEP and TLN is the presence of an additional arginine residue in the former. NEP Arg^{102} is believed to contribute to the peptidyl dipeptidase activity with short substrates by binding the free carboxyl group [28,29].

Crystallization of TLN in the presence of the NEP inhibitors, thiorphan and retrothiorphan, and determination of the complex structures by X-ray diffraction showed that Asn^{112} forms two hydrogen bonds with the P_2' residue of the inhibitors [19]. Asn^{112} is part of the Asn^{111}-Asn^{112}-Ala^{113}-Phe^{114}-Trp^{115} sequence that makes a β-sheet which holds the substrate in the active site. Hydrophobic cluster analysis [30] showed that the sequence Val^{541}-Asn^{542}-Ala^{543}-Phe^{544}-Tyr^{545} of NEP has some homology with the Asn^{112}-containing sequence of TLN and can also form a β-sheet. In order to assess the role of the NEP Asn^{542}, we used site-directed mutagenesis to change it for either Gly or Gln. Our biochemical data and inhibition analysis suggest that Asn^{542} of

Abbreviations used: NEP, neprilysin; octyl glucoside, *N*-octyl β-D-glucopyranoside; retrothiorphan, {[(*R*)-1-(mercaptomethyl)-2-phenylethyl]amino}-3-oxopropanoic acid; TBS, Tris-buffered saline; thiorphan, *N-(S)*-2-(mercaptomethyl)-1-oxo-3-phenylpropylglycine; TLN, thermolysin.

‡ To whom correspondence should be addressed.

NEP is involved in substrate binding by forming a unique hydrogen bond with the NH group of the P_2' residue.

EXPERIMENTAL

Site-directed mutagenesis and vector construction

All DNA manipulations were performed using previously described procedures [31]. Oligonucleotide-directed mutagenesis was performed by the method of Taylor et al. [32], with an M13 subclone containing the appropriate NEP cDNA fragment. Recombinant M13-NEP phages carrying the mutations were screened directly by DNA sequencing [33]. A DNA fragment containing the mutated region was isolated from the replicating form of the M13-NEP recombinant phage and substituted for the equivalent non-mutated fragment in pSVENK19, a previously described NEP expression vector [34]. The presence of the mutations and the absence of fortuitous changes in the expression vectors were confirmed by sequencing the entire coding region of the NEP cDNA by the chain-termination method for double-stranded templates using T7 DNA polymerase [35].

Transfection of COS-1 cells and binding of ^{125}I-2B12 monoclonal antibody

Non-mutated and mutated enzymes were produced by transient expression in COS-1 cells [36] using the standard calcium phosphate co-precipitation procedure [37] as previously described [38]. At 2 days after transfection, confluent cells were harvested by scraping into 1 ml of Tris-buffered saline (TBS; 10 mM Tris, pH 7.4, 150 mM NaCl) with a rubber spatula. Binding of ^{125}I-2B12 monoclonal antibody was measured on approx. 4×10^5 intact transfected cells as described previously [34], and was used to determine the relative amounts of NEP expressed at the cell surface.

Enzyme assays and immunoblotting

NEP from transfected COS-1 cells was solubilized in 50 mM Mes/NaOH, pH 6.5, containing 1 % octyl glucoside and enzyme activity measured essentially as described previously [22] using 50 nM tritiated substrate [*tyrosyl*-3,5-^{3}H][D-Ala^2][leucine]enkephalin (50 Ci/mmol) purchased from Research Products International Inc., except that incubations were carried out in 50 mM Mes/NaOH, pH 6.5, at 37 °C. K_m values were determined by the isotope-dilution method. Calculations were performed using the program ENZFITTER. In order to calculate $k_{cat.}$ values, enzyme was quantified by immunoblot analysis using purified rabbit kidney NEP as standard [22]. Immunoblotting was performed as previously described using monoclonal antibody 18B5, which recognizes a linear epitope of the rabbit NEP sequence (N. Labonté and P. Crine, unpublished work). The inhibitory effects of thiorphan [*N*-(DL-2-benzyl-3-mercaptopropionyl)glycine, Sigma Chemical Co.] or substituted thiorphan (synthesized in the laboratory of B.P. R.) were determined by serial dilution of the inhibitor in enzyme assays at a substrate concentration of 50 nM. IC_{50} values were calculated using the Sigma plot program.

RESULTS

Alignment of amino acid sequences of NEP, TLN and other related metallopeptidases

In the three-dimensional structures of the bacterial neutral proteases, TLN [11], neutral endoprotease [39,40] and elastase [41,42], there is an important β-sheet near the Zn^{2+}-binding site involved in substrate binding. This β-sheet acts as a 'jaw' forming important hydrogen bonds with the C-terminal residues of substrates or peptide-like inhibitors [43]. Sequence alignment of these bacterial (proteases) and mammalian neutral proteases (NEP, lactococcal endopeptidase, endothelin-converting enzyme and Kell-blood-group protein) shows a high similarity in a sequence located near the HEXXH consensus motif (Figure 1). Moreover in all sequences there is a conserved Asn residue which in TLN (Asn^{112}), as suggested by X-ray diffraction, forms two hydrogen bonds with the substrate [19]. This observation suggests that Asn^{542} in NEP may be the equivalent of Asn^{112} in TLN. If Asn^{542} in NEP is important for substrate binding, mutations at position 542 should result in decreased affinity of the mutated enzyme for its substrate. To test this hypothesis, we used site-directed mutagenesis to substitute Gly or Gln for Asn^{542}.

TLN	111	N	N	A	F	W	N	---25---	HEXXH
NAE	111	N	N	A	F	W	N	---24---	HEXXH
PAE	111	E	N	A	Y	W	N	---24---	HEXXH
NEP	541	V	N	A	F	Y	S	---37---	HEXXH
PepO	433	V	N	A	Y	Y	S	---37---	HEXXH
KELL	539	V	N	A	Y	Y	S	---37---	HEXXH
ECE	554	V	N	A	Y	Y	S	---37---	HEXXH

Figure 1 Alignment of amino acid sequences of TLN, NEP and other related metallopeptidases

Sequences around the Zn^{2+}-binding consensus (HEXXH) of TLN, neutral endoprotease (NAE) [39,40], elastase (PAE) [41,42], NEP, lactococcal endopeptidase (PepO) [44], Kell-blood-group protein (KELL) [45], and endothelin-converting enzyme (ECE) [46] are shown. Numbers beside residues indicate their positions in the respective sequences. Numbers above dashed lines indicate the distance between the short sequence alignment and the Zn^{2+}-binding consensus. The shaded box highlights a conserved asparagine residue.

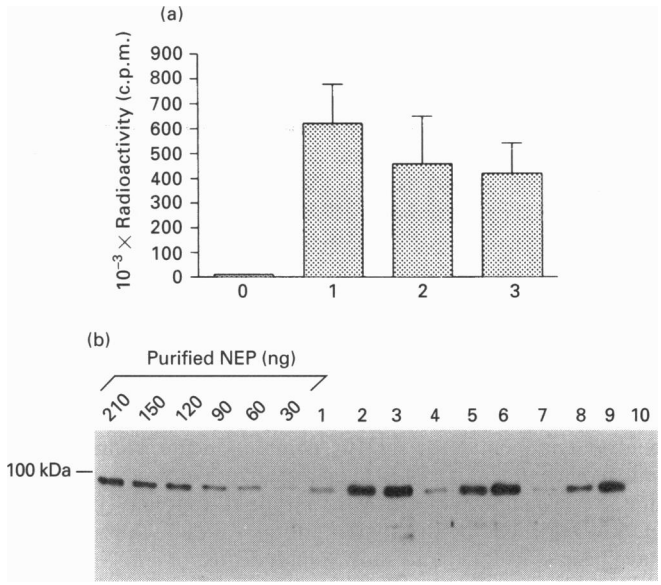

Figure 2 Expression of NEP proteins

(**a**) Binding assay of ^{125}I-2B12 monoclonal antibody to intact transfected cells. Transfections and radiolabelled-antibody-binding assay were performed as described in the Experimental section. 0, Mock-transfected COS-1 cells; 1, non-mutated NEP; 2, N542G mutant; 3, N542Q mutant. (b) Quantification of solubilized proteins by immunoblot analysis. Different amounts of purified rabbit kidney NEP were used as standard. Lanes 1–3, 2, 3 and 6 μl of non-mutated NEP; lanes 4–6, 2, 3 and 6 μl of N542G mutant; lanes 7–9, 2, 3 and 6 μl of N542Q mutant; lane 10, 6 μl of mock-transfected COS-1 cells. A molecular-mass standard is indicated on the left.

Table 1 Kinetic parameters of wild-type and mutated NEP

$\Delta\Delta G = -RT \ln [(k_{cat.}/K_m)$ mutated NEP$/(k_{cat.}/K_m)$ wild-type NEP]. Values are means ± S.E.M. of three different experiments.

	K_m (μM)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ (min^{-1}/μM)	$\Delta\Delta G$ (kJ/mol)
Wild-type	66 ± 5	1694 ± 54	25.7	–
N542G	912 ± 15	814 ± 65	0.893	8.8
N542Q	1077 ± 93	594 ± 24	0.551	10.0

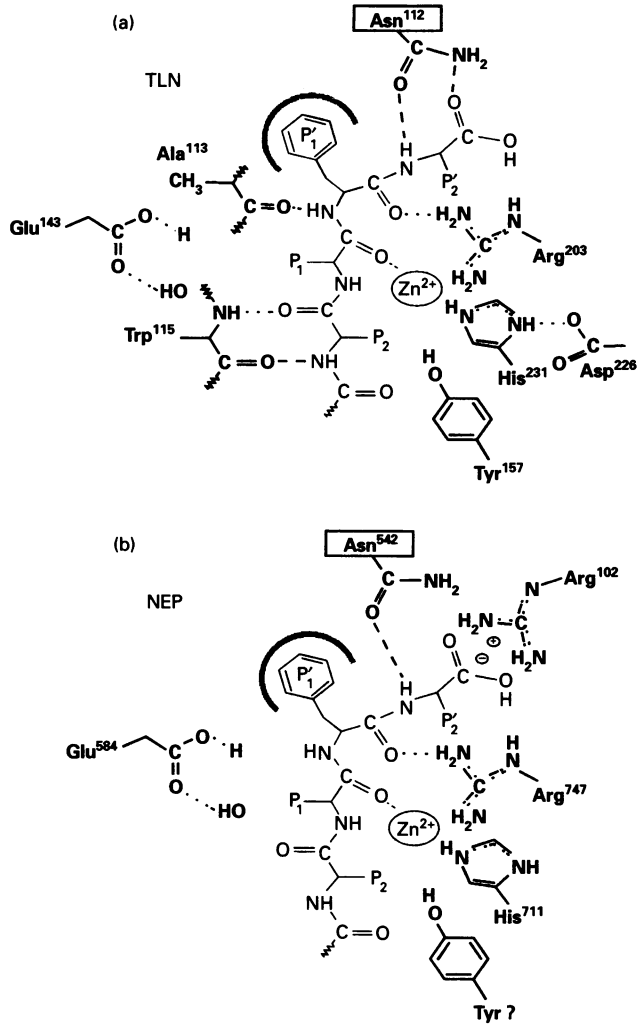

Figure 3 Schematic representations of substrate main chain binding with (a) TLN model and (b) putative NEP active site (based on TLN model)

Substrate main chain is in the centre of each model and residues involved in the substrate binding or in the catalysis are numbered. The Zn^{2+}-binding site is highlighted by a black circle and the hydrophobic pocket is shown by a black half-circle. Notice that, in NEP, Asn[542] forms a unique hydrogen bond with the N-terminal sites of P_2' and that the C-terminal site is stabilized by Arg[102]. In TLN the N- and C-terminal sites of P_2' are both hydrogen-bonded to Asn[112] (there is no Arg[102] counterpart in the TLN sequence).

Expression of native and mutated NEP in COS-1 cells

To obtain mutated and non-mutated enzymes, COS-1 cells were transfected with either vector pSVENK19 carrying the wild-type cDNA [34] or vectors pSVENK19-Gly 542 or pSVENK19-Gln 542 encoding N542G and N524Q NEP mutants respectively. The presence of the enzymes at the cell surface was monitored by either measuring the binding of monoclonal antibody ^{125}I-2B12, which recognizes a conformational epitope in the NEP ectodomain [47], or immunoblot analysis with a polyclonal antibody of proteins extracted from transfected COS-1 cells [48] (Figures 2a and 2b respectively). In all cases, similar amounts of wild-type and mutated enzymes were detected at the cell surface by the antibody-binding assay. Moreover, immunoblotting showed that the two mutated enzymes have the same apparent molecular mass as the non-mutated enzyme. Taken together, these results indicate that the mutations did not interfere with the biosynthesis and transport of the peptidases.

Table 2 Schematic representation of thiorphan and substituted thiorphans in putative NEP active site and IC_{50} values for wild-type NEP and N542G mutant

Results are means ± S.E.M. of different experiments.

	IC$_{50}$ (nM) Wild-type	N542G
Thiorphan	5.2 ± 0.6	30.2 ± 3.3
S448	287 ± 12	241 ± 5.3
S450	13.7 ± 1.6	69.6 ± 4.6
S455	597 ± 50	293 ± 19

Enzymic properties of NEP and the N542G and N542Q mutants

With [*tyrosyl*-3,5-^{3}H][D-Ala^2][Leu]enkephalin as substrate, NEP acts as a peptidyl dipeptidase, cleaving the pentapeptide between Gly^3 and Phe^4. To determine kinetic parameters of mutated and non-mutated NEP, we measured the rate of hydrolysis of this tritiated substrate. Mutations at position 542 resulted in an increase in K_m values (14–16-fold) whereas $k_{cat.}$ values were only slightly affected (2–3-fold decrease) (Table 1). The specificity factor $k_{cat.}/K_m$ was 29- and 47-fold lower than for the wild-type enzyme, for N542G and N542Q respectively. The change in free energy ($\Delta\Delta G$) for substrate binding, calculated from $\Delta\Delta G = -RT \ln[(k_{cat.}/K_m)$ mutated NEP$/(k_{cat.}/K_m)$ wild-type NEP] [49] was around 8–10 kJ/mol for both mutants (Table 1). This value suggests that Asn[542] of NEP contributes one or two hydrogen bonds to substrate binding [50].

Inhibition of NEP and N542G mutant

In TLN, Asn^{112} forms two hydrogen bonds with the NH and CO groups of the P_2' residue (Figure 3a). To determine if Asn^{542} of NEP interacts with the same groups, we studied the inhibition of wild-type and N542G NEP with thiorphan or substituted thiorphan inhibitors (Table 2). The substituted inhibitors have methyl groups replacing the proton of the NH group and/or the carboxyl group of the P_2' residue. Thus no hydrogen bond can be formed at these positions.

The IC_{50} of thiorphan for N542G was approx. 6-fold higher than that for the wild-type enzyme (Table 2). This result supports our previous conclusion that Asn^{542} of NEP is involved in substrate and inhibitor binding. Accordingly, replacing the proton of the NH group of the P_2' residue by a methyl group (inhibitor S448) resulted in an approx. 60-fold increase in the IC_{50} of the inhibitor compared with thiorphan (Table 2). As expected, inhibitor S448 had a similar IC_{50} for NEP and for N542G. Replacing the carboxyl group of the P_2' residue of thiorphan by a methyl group (inhibitor S450) resulted in a smaller increase in the IC_{50} (2.5-fold). This effect was seen with both non-mutated and mutated enzyme. Finally, when the changes assessed in S448 and S450 were simultaneously introduced (inhibitor S455), an additive effect on the IC_{50} was observed and this effect is more important for the wild-type enzyme (Table 2).

DISCUSSION

Crystal-structure comparisons between TLN, neutral endoprotease and elastase show a 0.2 nm (2Å) movement of a β-sheet strand (residues 112–116 of TLN) that can act as a 'jaw' to hold the substrate in the active site [43]. In particular, Asn^{112} of TLN forms two hydrogen bonds with the P_2' residue of the substrate. Sequence alignment and hydrophobic cluster analysis [30] identified a corresponding NEP sequence (residues 542–545; see Figure 1) in which Asn^{542} may possibly be the counterpart of Asn^{112} of TLN and thus be involved in substrate binding. Mutation of this NEP Asn residue to Gly or Gln causes augmentation of the Michaelis constant but has little impact on $k_{cat.}$ values. Furthermore, the observation that Gln, which has one more methylene group(s) in its side chain, cannot replace Asn underlines the importance of the correct positioning of the amide function of Asn^{542} for interaction with the substrate. Consequently, these results strongly suggest that Asn^{542} of NEP, like Asn^{112} of TLN, is involved in substrate binding.

In TLN, Asn^{112} contributes two hydrogen bonds to substrate binding: one with the NH group of the P_2' residue and one with the CO group of the same residue (Figure 3a). The calculated changes in free energy ($\Delta\Delta G$) for NEP mutants suggest that Asn^{542} is involved in substrate binding, forming a unique or two weak hydrogen bond(s) [50]. This was further investigated by using thiorphan or substituted thiorphan inhibitors. The IC_{50} of thiorphan for N542G was 6-fold higher than that for the non-mutated enzyme, supporting the conclusion, drawn from the measurement of kinetic parameters, that Asn^{542} is involved in substrate and inhibitor binding. This decrease in affinity of N542G for this inhibitor is smaller than that observed for the substrate. The best explanation for this observation is that a large proportion of the binding energy of the inhibitor is provided by the interaction of the sulphur atom, a strong metal-co-ordinating group, with the active-site Zn^{2+} atom [8,16].

Inhibitor S448, in which the proton of the NH group of the P_2' residue is replaced by a methyl group, showed an approx. 60-fold increase in IC_{50} for NEP as compared with thiorphan. This increase is less for the N542G mutant, probably because of the presence of the bulkier methyl group in the inhibitor structure which causes a stronger steric hindrance to the asparagine side chain in NEP than to the glycine in the mutant. Furthermore, the observation that both wild-type enzyme and N542G mutant are inhibited by S448 with similar IC_{50} values is consistent with our hypothesis that the NH proton of the P_2' residue interacts with Asn^{542}. All these results are consistent with the findings of other authors who observed that the replacement of the hydrogen from the NH group of the P_2' residue by a methyl or cyclopropyl group produced a dramatic loss of inhibitor binding [51–55].

On the other hand, inhibitor S450, in which the carboxyl group of the P_2' residue has been replaced by a methyl group, showed a smaller but similar increase in the IC_{50} value for both NEP and the mutant N542G, suggesting that the carboxyl of P_2' does not interact with Asn^{542} of NEP. This result is consistent with the earlier studies of Fournié-Zaluski et al. [51] on the weaker inhibitory potencies of enkephalins substituted at the C-terminal position, suggesting that NEP may function as a peptidyl dipeptidase through ionic interaction with the free carboxyl group of the substrate. This was firmly confirmed by the work of Beaumont et al. [27] and Bateman et al. [28] showing that the carboxyl group of short substrates interacts with Arg^{102} of NEP instead (Figure 3b). Finally, inhibitor S455 confirms the importance of both interactions (NH and CO of the P_2' moiety) in binding inhibitors. The smaller impact on the N542G mutant can be explained by the replacement of Asn^{542} by Gly which probably reduces the steric hindrance caused by the *N*-methyl group to the asparagine side chain in the NEP active site.

Considering these data, we propose that Ans^{542} forms a unique hydrogen bond with the NH proton of the P_2' residue. This interaction, along with the interaction of Arg^{102} with the carboxyl group, stabilizes the carboxyl end of short substrates and thus may contribute to the peptidyl dipeptidase activity of NEP (Figure 3b). However, we cannot rule out the possibility that, with longer substrates, NEP Asn^{542} establishes two hydrogen bonds with the P_2' residue, as does Asn^{112} in TLN. Further experiments are required to test this hypothesis.

In conclusion, the specificity of Zn^{2+} metalloproteases/peptidases is first determined by van der Waals and ionic interactions between their subsites and the side chains of the amino acid residues of the substrate. The stability of the bound substrate is increased by several well-positioned hydrogen bonds between donor and acceptor groups of the bound molecule and the polar residues of the peptidases. In the present study we identified an important hydrogen bond in NEP between Asn^{542} and the NH group of the P_2' residue involved in the holding of a bound substrate and/or inhibitor.

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REFERENCES

1 Kenny, A. J., Stephenson, S. L. and Turner, A. J. (1987) in Cell Surface Peptidase in Mammalian Ectoenzymes (Kenny, A. J. and Turner, A. J., eds.), pp. 169–210, Elsevier, Amsterdam
2 Hooper, N. M. (1993) in Ectopeptidases in Barriers to Protein Delivery (Audus, K. N. and Raub, T. J., eds.), Plenum Press, New York
3 Malfroy, B., Kuang, W. J., Seeburg, P. H., Mason, A. J. and Schofield, P. R. (1988) FEBS Lett. **229**, 206–210
4 Devault, A., Lazure, C., Nault, C. et al. (1987) EMBO J. **6**, 1317–1322
5 Malfroy, B., Schofield, P. R., Kuang, W. J., Seeburg, P. H., Mason, A. J. and Henzel, W. J. (1987) Biochem. Biophys. Res. Commun. **144**, 59–66
6 Chen, C. Y., Salles, G., Seldin, M. F., Kister, A. E., Reinherz, E. L. and Shipp, M. A. (1992) J. Immunol. **148**, 2817–2825

7 Relton, J. M., Gee, N. S., Matsas, R., Turner, A. J. and Kenny, A. J. (1983) Biochem J. **215**, 519–523
8 Roques, B. P., Noble, F., Daugé, V., Fournié-Zaluski, M.-C. and Beaumont, A. (1993) Pharmacol. Rev. **45**, 87–146
9 Rawlings, N. D. and Barrett, A. J. (1993) Biochem J. **290**, 205–218
10 Blundell, T. M. (1994) Nat. Struct. Biol. **1**, 73–75
11 Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A. and Neurath, H. (1972) Nature (London) New Biol. **238**, 41–43
12 Hooper, N. M. (1994) FEBS Lett. **354**, 1–6
13 Suda, H., Aoyagi, T., Takeuchi, T. and Umezawa, H. (1973) J. Antibiot. **26**, 621–623
14 Fulcher, I. S., Matsas, R., Turner, A. J. and Kenny, A. J. (1982) Biochem. J. **203**, 519–522
15 Matthews, B. W. (1988) Acc. Chem. Res. **21**, 333–340
16 Roques, B. P., Fournié-Zaluski, M.-C., Soroca, E. et al. (1980) Nature (London) **288**, 286–288
17 Roques, B. P., Lucas-Soroca, E., Chaillet, P., Costentin, J. and Fournié-Zaluski, M.-C. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3178–3182
18 Benchetrit, T., Fournié-Zaluski, M.-C. and Roques, B. P. (1987) Biochem. Biophys. Res. Commun. **147**, 1034–1040
19 Roderick, S. L., Fournié-Zaluski, M.-C., Roques, B. P. and Matthews, B. W. (1989) Biochemistry **28**, 1493–1497
20 Jongeneel, C. V., Bouvier, J. and Bairoch, A. (1989) FEBS Lett. **242**, 211–214
21 Devault, A., Sales, N., Nault, C., Beaumont, A., Roques, B. P., Crine, P. and Boileau, G. (1988) FEBS Lett. **231**, 54–58
22 Le Moual, H., Devault, A., Roques, B. P., Crine, P. and Boileau, G. (1991) J. Biol. Chem. **266**, 15670–15674
23 Le Moual, H., Dion, N., Roques, B. P., Crine, P. and Boileau, G. (1994) Eur. J. Biochem. **221**, 475–480
24 Bateman, R. C., Jr., Young, A. E., Slaughter, C. and Hersh, L. B. (1990) J. Biol. Chem. **265**, 8365–8368
25 Dion, N., Le Moual, H., Crine, P. and Boileau, G. (1993) FEBS Lett. **318**, 301–304
26 Vijayaraghavan, J., Kim, Y.-A., Jackson, D., Orlowski, M. and Hersh, L. B. (1990) Biochemistry **29**, 8052–8056
27 Beaumont, A., Le Moual, H., Boileau, G., Crine, P. and Roques, B. P. (1991) J. Biol. Chem. **266**, 214–220
28 Bateman, R. C., Jr., Jackson, D., Slaughter, C. A. et al. (1989) J. Biol. Chem. **264**, 6151–6157
29 Beaumont, A., Barbe, B., Le Moual, H. et al. (1992) J. Biol. Chem. **267**, 2138–2141
30 Benchetrit, T., Bissery, V., Mornon, J. P., Devault, A., Crine, P. and Roques, B. P. (1988) Biochemistry **27**, 592–596
31 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
32 Taylor, J. W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. **13**, 8765–8785
33 Sanger, F., Niklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3963–3965
34 Devault, A., Nault, C., Zollinger, M., Fournié-Zaluski, M.-C., Roques, B. P., Crine, P. and Boileau, G. (1988a) J. Biol. Chem. **263**, 4033–4040
35 Tabor, S. and Richardson, C. C. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4767–4771
36 Gluzman, Y. (1981) Cell **23**, 175–182
37 Graham, F. L. and Van der Eb, A. J. (1973) Virology **52**, 456–467
38 Noél, G., Zollinger, L., Larivière, N., Nault, C., Crine P. and Boileau, G. (1987) J. Biol. Chem. **262**, 1876–1881
39 Pauptit, R. A., Karlson, R., Picot, D., Jenkins, J. A., Niklaus-Reimer, A. S. and Jansonius J. N. (1988) J. Mol. Biol. **199**, 525–537
40 Stark, W., Pauptit R. A., Wilson, K. S. and Jansonius, J. N. (1992) Eur. J. Biochem. **207**, 781–791
41 Thayer, M. M., Flaherty, K. M. and McKay, D. B. (1991) J. Biol. Chem. **266**, 2864–2871
42 McKay, D. B., Thayer, M. M., Flaherty, K. M., Pley, H. W. and Benvegnu, D. (1992) Matrix Supp. **1**, 112–115
43 Holland, D. R., Tronrud, D. E., Pley, H. W. et al. (1992) Biochemistry **31**, 11310–11316
44 Mierau, I., Tan, P. S. T., Haandrikman, A. J. et al. (1993) J. Bacteriol. **175**, 2087–2096
45 Lee, S., Zambas, E. D., Marsh, W. L. and Redman, C. M. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6353–6357
46 Xu, D., Emoto, N., Giaid, A., Slaughter, C., Kaw, S., deWit, D. and Yanagisawa, M. (1994) Cell **78**, 473–485
47 Aubry, M., Zollinger, M., Fortin, S., Venien, C., LeGrimellec, C. and Crine, P. (1988) Biochem. Biophys. Acta **967**, 56–64
48 Lemay, G., Wasksman, G., Roques, B. P., Crine, P. and Boileau, G. (1989) J. Biol. Chem. **264**, 15620–15623
49 Wilkinson, A. J., Fersht, A. R., Blow, D. M. and Winter, G. (1983) Biochemistry **22**, 3581–3586
50 Fersht, A. R., Shi, J.-P., Knill-Jones, J. et al. (1985) Nature (London) **314**, 235–238
51 Fournié-Zaluski, M.-C., Perdrisot, R., Gacel, G., Swertz, J. P., Roques, B. P. and Schwartz, J. C. (1979) Biochem. Biophys. Res. Commun. **91**, 130–135
52 Llorens, C., Gacel, G., Swerts, J. P. et al. (1980) Biochem. Biophys. Res. Commun. **96**, 1710–1716
53 Fournié-Zaluski, M.-C., Lucas, E., Waskman, G. and Roques, B. P. (1984) Eur. J. Biochem. **139**, 267–274
54 Xie, J., Soleichac, J. M., Schmidt, C., Peyroux, J., Roques, B. P. and Fournié-Zaluski, M.-C. (1989) J. Med. Chem. **32**, 1497–1503
55 Mimura, T., Nakamura, Y., Nishino, J. et al. (1992) J. Med. Chem. **35**, 602–608

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