Differences in transition state stabilization between thermolysin (EC 3.4.24.27) and neprilysin (EC 3.4.24.11)

Cynthia Marie-Claire, Emmanuel Ruffet, Gilles Tiraboschi, Marie-Claude Fournie-Zaluski*

Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM, URA D1500 CNRS, UFR des Sciences Pharmaceutiques et Biologiques, Faculté de Pharmacie, 4, Avenue de l'Observatoire, 75270 Paris Cedex 06, France

Received 20 August 1998

Abstract Important homologies in the topology of the catalytic site and the mechanism of action of thermolysin and neprilysin have been evidenced by site-directed mutagenesis. The determination of differences in transition state stabilization between these peptidases could facilitate the design of specific inhibitors. Thus, two residues of thermolysin which could be directly (Tyr^{157}) or indirectly (Asp^{226}) involved in the stabilization of the transition state and their putative counterparts in neprilysin (Tyr^{625} and Asp^{709}) have been mutated. The results show that Tyr^{157} is important for thermolysin activity while Tyr^{625} has no functional role in neprilysin. Conversely, the mutation of Asp^{226} induced a slight perturbation of thermolysin activity while Asp^{709} in neprilysin seems crucial in neprilysin catalysis. Taken together these data seem to indicate differences in the transition state mode of stabilization in the two peptidases.

Key words: Neprilysin; Thermolysin; Site-directed mutagenesis; Molecular modeling

1. Introduction

Neprilysin (EC 3.4.24.11, NEP) is a member of the M13 family from the MA clan of zinc metallopeptidases [1]. In addition to the active site consensus sequence HExxH, where the histidines are two of the zinc ligands and the glutamate is involved in the catalytic mechanism, enzymes of the M13 family are characterized by a second consensus sequence ExxxD, where the glutamate is the third ligand of the zinc ion and the aspartate is involved in the stabilization of the active site [2]. Studies of the subsite specificity of neprilysin, using various substrates and inhibitors, have shown that this enzyme has significant analogies with the bacterial zinc metallopeptidase thermolysin (EC 3.4.24.27) [2].

Thermolysin, a thermostable neutral protease produced by the genus *Bacillus thermoproteolyticus* [3], belongs to the M4 family of the MA clan of zinc metallopeptidases. The co-crystallization of thermolysin with several inhibitors has made it possible to propose an enzymatic mechanism that has been generalized to all the enzymes of this clan (review in [4]). Neprilysin was first cloned in 1987 [5]. It is a type II membrane protein expressed in many tissues including kidney and central nervous system and is involved in the catabolism of active peptides such as the atrial natriuretic peptide and enkephalins (review in [2]). Neprilysin inhibitors are of great interest since they show anti-nociceptive and anti-hypertensive properties [2].

Despite a weak homology between the primary sequences of these two enzymes, there are significant secondary structure similarities between thermolysin and the C-terminal portion of neprilysin [6]. This allowed the identification by site-directed mutagenesis of several residues in the active site of neprilysin. Among those the three zinc ligands have been identified as His^{583}, His^{587} and Glu^{646} (His^{142}, His^{146} and Glu^{166} in thermolysin) [7,8]. Glu^{584} (Glu^{143} in thermolysin) was proposed to be involved in catalysis [9] by polarizing the attacking water molecule. His^{711} (His^{231} in thermolysin) has been shown to be involved in the stabilization of the transition state [10]. Asp^{650} (Asp^{170} in thermolysin) belongs to the second consensus sequence [11] and is H bonded to both His^{583} (His^{142} in thermolysin) and Arg^{717} (Arg^{203} in thermolysin) [12]. Moreover, these residues are involved in a large hydrogen bond network important for the stabilization of the active site and catalytic activity [12]. These results have been used to build a three-dimensional model of the C-terminal domain of neprilysin, which includes the active site of the enzyme. Fig. 1 is a schematic representation of the active site of thermolysin according to the crystallographic data, the corresponding residues in the reconstituted active site of neprilysin are given in italics. In this model, based on sequence alignments, it has been suggested that Tyr^{625} of neprilysin could have a role similar to that of Tyr^{157} of thermolysin which is proposed to be involved in the stabilization of the transition state [4] (Fig. 1). Moreover, Asp^{709} of neprilysin seems to be the counterpart of Asp^{226} in thermolysin, which participates in the stabilizing hydrogen bond network by interacting with His^{231} (Fig. 1).

To verify these hypotheses and improve the characterization of neprilysin's mechanism of action, comparative mutations were performed on both enzymes: Tyr^{157} of thermolysin and Tyr^{625} of neprilysin were mutated to phenylalanine, and Asp^{226} of thermolysin and Asp^{709} of neprilysin to alanine. The enzymatic and inhibitor binding properties of the mutants were analyzed in terms of homologies or differences in their structures and mechanisms of action. The results were also compared to those obtained previously in enzymes belonging to other families of zinc metallopeptidases.

2. Materials and methods

2.1. Materials

Restriction and modifying enzymes, bacterial culture media and the QuickChange Site Directed Mutagenesis kit were respectively from New England Biolabs, Bio 101 and Stratagene obtained from Ozyme (France). The Thermo Sequenase enzyme (United States Biochemical) and [α-^{33}P]ddNTP was purchased from Amersham (France). Cell cul-

*Corresponding author. Fax: (33) (1) 43-26-69-18.
E-mail: roques@pharmacie.univ-paris5.fr

Abbreviations: Phosphoramidon, *N*-(α-rhamnopyranosyl-(oxyhydroxyphosphinyl))-L-leucyl-1-tryptophan; thiorphan, *N*-(2-(*R*,*S*)-mercaptomethyl)-1-oxo-3-phenylpropyl)glycine; ECE, endothelin converting enzyme; HACBO-Gly, *N*-(2-(*R*,*S*)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl)-glycine

PII: S0014-5793(98)01267-8

ture products were from Life Technologies (France). mAbs Alb1 and T1 were generous gifts from Dr. C. Boucheix (Villejuif, France) and Dr. C. Bohuon (Villejuif, France) respectively. Oligonucleotides were synthesized by Genosys (UK). Leucine enkephalin (tyrosyl-glycyl-glycyl-phenylalanyl-leucine), D-Ala^2-leucine enkephalin (tyrosyl-D-alanyl-glycyl-phenylalanyl-leucine) and glycyl-D-phenylalanine (Gly-D-Phe) were purchased from Bale Biochimie (France). [^{3}H]Leucine enkephalin and [^{3}H]D-Ala^2-leucine enkephalin were purchased from Izinta (Hungary). CNBr-activated Sepharose 4B resin was purchased from Pharmacia (France) and bestatin, phosphoramidon and captopril were from Sigma (France). The inhibitors thiorphan [13] and HACBO-Gly [14] were prepared in the laboratory following reported procedures.

2.2. Vector construction and site-directed mutagenesis

The *Bacillus* strain used was *B. subtilis* DB117, lacking neutral protease activity [15]. The previously described pTLN-Mat3 plasmid [12] encoding the sequence of the mature enzyme was used for double strand mutagenesis in *Escherichia coli*. The mutated sequence *Eco*RI/*Mlu*I was then subcloned in pTLN2 encoding the full length of the enzyme to express the enzymes in *B. subtilis* DB117 as described before [16]. For plasmid transformation, *B. subtilis* protoplasts were prepared as described [17]. Purification of large-scale plasmid preparations was achieved using a modified polyethylene glycol precipitation method [18]. The plasmid pcDNA-NEP has been previously described [12].

Double strand mutagenesis was carried out using the QuickChange Site Directed Mutagenesis kit following the manufacturer's instructions. Oligonucleotides were designed to replace Asp^{226} in thermolysin and Asp^{709} in neprilysin by an alanine, and Tyr^{157} in thermolysin and Tyr^{625} in neprilysin by a phenylalanine. The authenticity of each mutation was confirmed by sequencing the complete coding sequence.

2.3. Expression and purification of wild type and mutated thermolysins

B. subtilis DB117 cells harboring the plasmid pTLN2 were grown in L-Broth containing 5 mM $CaCl_2$ and 5 μg/ml chloramphenicol, at 37°C with shaking (180 rpm) for 16 h. Wild type and mutated enzymes were purified from the culture supernatant by affinity chromatography using a column of Gly-D-Phe, coupled to CNBr-activated Sepharose 4B resin as described previously [16].

2.4. Cell culture and transfection

COS cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 50 μg/ml gentamicin at 37°C. One day before transfection cells were seeded into 10 cm Petri dishes, at a density of 10^6 cells/dish. Cells were transfected with the pcDNA-NEP vector encoding wild type or mutant neprilysin, using a DEAE-dextran-chloroquine method [19]. 48 h after transfection, cells were solubilized at a protein concentration of 1 mg/ml in 50 mM Tris-HCl, pH 7.4 containing 1% (w/v) *n*-octyl glucoside for 30 min on ice. The preparation was then centrifuged at 20 000 × *g* for 15 min and the supernatant was used for enzymatic assays.

2.5. Western blotting

Proteins were subjected to 8% (neprilysin) or 12% (thermolysin) SDS-PAGE and blotted onto nitrocellulose filters. The blots were sequentially incubated with mAb Alb1 raised against human neprilysin or mAb T1 raised against thermolysin and with an anti-mouse Ig, horseradish peroxidase-linked antibody (Amersham, France). The peroxidase activity was revealed with the ECL detection kit from Amersham (France).

2.6. Enzyme assays

Thermolysin activity and K_i values of inhibitors were determined as previously described [16,20] using 20 nM of [^{3}H]leucine enkephalin as a substrate. K_m and k_{cat} values were determined by the isotopic dilution method. Neprilysin activity and K_i values of inhibitors were measured as previously described using 40 nM [^{3}H]D-Ala^2-leucine enkephalin as substrate [21]. K_m values were determined by the isotopic dilution method. In both cases calculations were done using the program ENZFITTER (Biosoft). In order to determine the k_{cat} values the neprilysin levels were evaluated by dot blot using recombinant human neprilysin as standard (generously provided by Khepri Pharmaceuticals, USA) [12]. IC_{50} values were considered to be equal to K_i values as the concentrations of substrates used were much lower than K_m values. In all cases the reactions were stopped when substrate degradation was ≤10%.

3. Results

3.1. Expression of wild type and mutated enzymes

Wild type and mutant thermolysins were purified from the culture supernatant of *B. subtilis* DB117 using a Gly-D-Phe affinity column with yields of approximately 5 mg/l for wild type enzyme, and 0.5 mg/l for D^{226}A and Y^{157}F. Thermolysin is expressed as a pre-proenzyme and we have previously shown that its maturation necessitates an autocatalytic and intramolecular cleavage of the precursor pro-thermolysin [22] accounting for the lower yields obtained in the case of the mutants. Western blot analysis, with mAb T1, showed an apparent molecular mass for the three enzymes of 36 kDa (Fig. 2A).

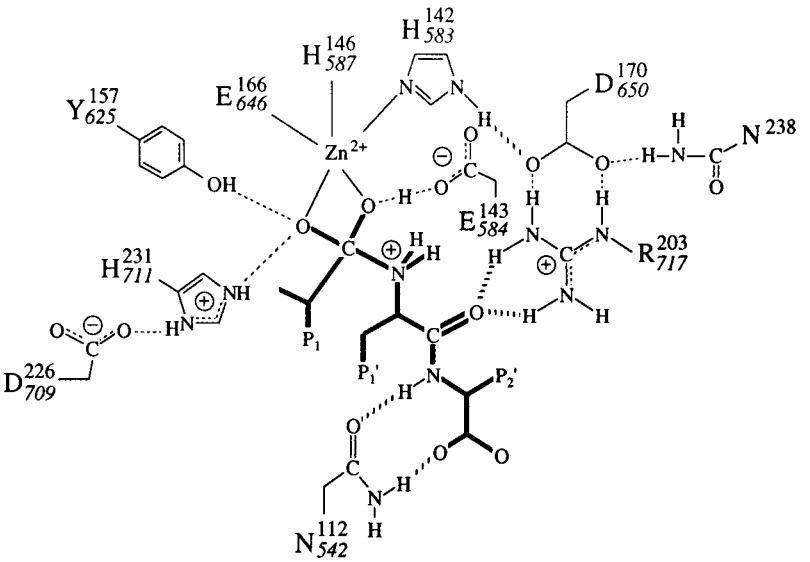


Fig. 1. Schematic representation of the active site of thermolysin. The apparent network of hydrogen bonds between the residues involved in catalysis is represented by broken lines. The numbers of the corresponding residues in the neprilysin active site are given in italics.

The wild type and mutated neprilysins were expressed in COS cells, and analyzed by immunoblotting. As seen in Fig. 2B, wild type, $D^{709}A$ and $Y^{625}F$ neprilysin migrated with the same apparent molecular mass (98 kDa) and had the same level of expression indicating that the mutation did not interfere with the biosynthesis of neprilysin. Correct glycosylation of the enzymes was verified by endoglycosidase H and *N*-glycosidase F digestion (data not shown).

3.2. Kinetic parameters of the tyrosine mutants in thermolysin and neprilysin

Mutation $Y^{157}F$ in thermolysin affected mainly the k_{cat} value (63-fold diminution) (Table 1). The specific activity of this mutant is then reduced 92-fold as compared to that of the wild type enzyme. The proposed counterpart of this tyrosine in neprilysin is Tyr^{625}. When this residue was mutated to phenylalanine only a slight increase in the K_m value was observed with no change in the k_{cat} value (Table 1). The resulting k_{cat}/K_m ratio is 2.9-fold lower than that of the wild type enzyme.

3.3. Inhibition studies with the tyrosine mutants in thermolysin and neprilysin

In the case of the $Y^{157}F$ mutant in thermolysin the K_i value for the thiol inhibitor thiorphan is increased by a factor of 3.4 while that of the hydroxamate inhibitor HACBO-Gly is increased 10-fold. Since these two inhibitors differ only in the nature of the zinc ligand it seems that the effect observed may be due to the mode of binding to the zinc ion (monodentate with a thiol and bidentate for an hydroxamate). The K_i value of the transition state analogue phosphoramidon is increased by a factor of 3 similar to that of the 'collected product' analogue thiorphan. When Tyr^{625} was mutated to phenylalanine in neprilysin the only difference observed in the K_i values of the inhibitors tested was a 5.5-fold increase in the case of phosphoramidon (Table 2).

3.4. Kinetic parameters of the aspartate mutants in thermolysin and neprilysin

In the case of the $D^{226}A$ mutant in thermolysin the K_m value is increased by 9.4-fold and the k_{cat} is decreased 4-fold (Table 1). The k_{cat}/K_m ratio is decreased by 39-fold as compared to the wild type enzyme. When the proposed counterpart of this aspartate was mutated in neprilysin (Asp^{709}), the K_m value obtained was similar to that of the wild type enzyme while the k_{cat} value was dramatically decreased (a factor of 319). The resulting specific activity is thus decreased by a factor of 298 as compared to the wild type enzyme.

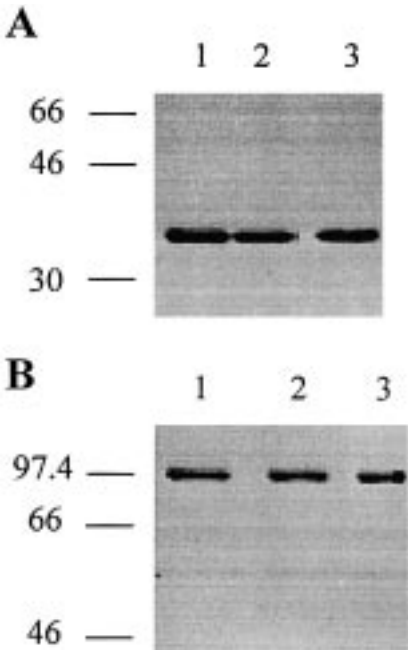


Fig. 2. Analysis of wild type and mutated enzymes by Western blots as described in Section 2. A: Lane 1, wild type thermolysin; lane 2, $Y^{157}F$ mutant; lane 3, $D^{226}A$ mutant. B: Lane 1, wild type neprilysin; lane 2, $Y^{625}F$ mutant; lane 3, $D^{709}A$ mutant.

3.5. Inhibition studies with the aspartate mutants in thermolysin and neprilysin

In the case of the $D^{226}A$ mutant of thermolysin the effects observed are an 8-fold and a 6-fold increase of the K_i values in the case of thiorphan and HACBO-Gly (Table 2). Interestingly, the K_i of phosphoramidon is only increased by a factor of 3. When the proposed counterpart of this aspartate was mutated in neprilysin the resulting mutant $D^{709}A$ showed dramatic decreases in the inhibitory potencies of the inhibitors tested (Table 2). The increase in K_i value was of 7.5×10^3 in the case of thiorphan and $>10^4$ in the case of phosphoramidon. Interestingly, when the zinc ligand is a hydroxamate (HACBO-Gly) the factor is only 3.6.

4. Discussion

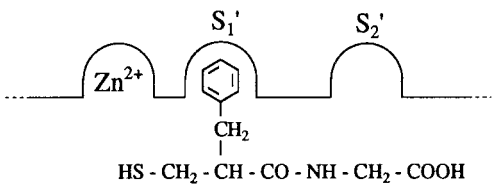
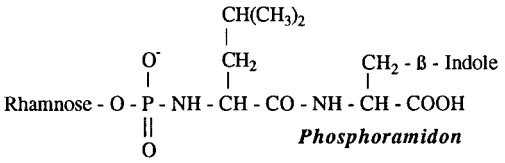
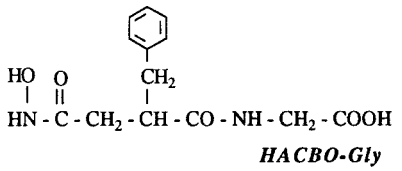
In order to design new potent and selective inhibitors of neprilysin it is important to have a precise idea of its active site organization. In the absence of crystallographic data on this enzyme site-directed mutagenesis studies, in association

Table 1
Kinetic constants for the hydrolysis of leucine enkephalin by wild type, $Y^{157}F$ and $D^{226}A$ thermolysins and for the hydrolysis of D-Ala^2-Leucine enkephalin by wild type, $Y^{625}F$ and $D^{709}A$ neprilysins

	Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
Thermolysin	Wild type	210 ± 25	9329 ± 754	44.4
	$Y^{157}F$	480 ± 26	100 ± 2	0.21
	$D^{226}A$	1985 ± 54	2252 ± 87	1.13
Neprilysin	Wild type	55 ± 6	1149 ± 144	20.9
	$Y^{625}F$	141 ± 14	1003 ± 46	7.1
	$D^{709}A$	47 ± 6	3.6 ± 0.27	0.07

Reactions were carried out as described in Section 2. Data are means ± S.E.M. from at least three independent determinations.

Table 2
K_i values of inhibitors for the wild type and mutated thermolysins and neprilysins[a]

	Thermolysin, Ki (μM)			Neprilysin, Ki (nM)		
	Sauvage	Y157F	D226A	Sauvage	Y625F	D709A
 *Thiorphan*	1.6 ± 0.2	7.6 ± 0.5	13.0 ± 0.1	2.4 ± 0.8	2.9 ± 0.5	18,000 ± 2,000
 *Phosphoramidon*	0.06 ± 0.01	0.031 ± 0.001	0.19 ± 0.04	0.8 ± 0.2	4.4 ± 0.2	> 1,000,000
 *HACBO-Gly*	3.1 ± 0.2	31 ± 5	19 ± 2	1.4 ± 0.2	2.6 ± 0.5	5.1 ± 0.5

[a]Data are means ± S.E.M. from at least three independent determinations.

with molecular modeling, remain useful tools to investigate the functional roles of specific residues in the neprilysin active site. Thermolysin is the model of active site for neprilysin and previous studies have already proved the similarities between the two active sites [7–12]. In this work we studied residues implicated directly or not in the transition state stabilization of these two enzymes. Tyr^{157} is proposed to be implicated, with His^{231}, in the stabilization of the transition state as deduced from the crystallographic structure of thermolysin-inhibitor complexes [4] and Tyr^{625} in neprilysin has been proposed to be the counterpart of Tyr^{157} in thermolysin according to sequence alignments [23]. The two tyrosines were mutated to phenylalanine in order to alter as little as possible the organization of the active sites. The recent achievement of a three-dimensional model of neprilysin active site (Tiraboschi, personal communication) makes it possible to propose Asp^{709} of neprilysin as the equivalent of Asp^{226} of thermolysin which stabilizes His^{231} by a hydrogen bond. In both enzymes the aspartate residues were mutated to alanine to abolish this interaction.

The decrease in k_{cat}/K_m value observed with the mutant $Y^{157}F$ in thermolysin (200-fold) is consistent with the crystallographic data and the proposed role of this residue in the stabilization of the transition state [4]. His^{231} was also implicated in the stabilization of the transition state [4]. However, when this residue was mutated in thermolysin [24], a dramatic 500-fold decrease in the k_{cat} value was observed suggesting that the role of Tyr^{157} in the stabilization of the transition state is slightly less important than that of His^{231}. Moreover, the effects observed on the K_i values of the inhibitors tested were smaller than those obtained with His^{231}. In the case of the mutation of His^{231} in phenylalanine in thermolysin the decrease in inhibitory potency of phosphoramidon was $> 10^4$ [24]. This difference may be consistent with a minor role of Tyr^{157} in the stabilization of the transition state as compared to His^{231}. It could be observed that in the crystallized complexes of thermolysin with inhibitors mimicking the transition state, His^{231} is involved in all cases in the stabilization of this intermediate, but Tyr^{157} is found in only some examples such as phosphoramidon, $ZF^{P}LA$ or CLT [4]. This seems to indicate that this interaction is not predominant for the binding of these inhibitors. This was confirmed here by the slight modification observed in the K_i value of phosphoramidon in the $Y^{157}F$ mutant enzyme. However, the important perturbation induced by this mutation in the catalytic process indicates that the inhibitors crystallized to date with thermolysin could represent structural models for different steps in the catalysis, but are not 'real' intermediates.

When Tyr^{625} was mutated to phenylalanine in neprilysin the kinetic parameters and K_i values of the inhibitors tested were similar to those of the wild type enzyme. These results suggest that either this tyrosine is not the equivalent of Tyr^{157} in thermolysin or Tyr^{625} in neprilysin is equivalent to Tyr^{157} of thermolysin but does not have a role in the stabilization of the transition state. Sequence alignments of the neprilysins (Kell, ECE-1, ECE-2, PEX and Pepo) show that this tyrosine is conserved in all the enzymes except in Pepo where it is replaced by a phenylalanine [25–30]. This could indicate that this residue does not have a significant role in the M13 family of zinc metallopeptidases as compared to thermolysin. It is also interesting to compare these results with those obtained when the residues assumed to be the equivalents of Tyr^{157} were mutated in other zinc metallopeptidases. The mutation of Tyr^{683} in ECE-1 (personal communication), which is analogous to Tyr^{157} of thermolysin according to sequence alignments, showed that this residue does not play any role in the enzymatic activity of this enzymes as observed here for neprilysin. In the case of human testicular angiotensin converting enzyme (ACE) (Tyr^{200}) a slight effect has been reported on the k_{cat} while no effect was found in rabbit testicular ACE (Tyr^{236}) [31,32]. Conversely, the mutation of Tyr^{471} in APA [33] induced a large decrease in the k_{cat} value of the mutated

enzyme. Taken together these results seem to indicate that an evolution in the structure of the active sites, and consequently in the mechanism of action, of the different families of zinc metalloenzymes could have occurred. In thermolysin family two residues (His^{231} and Tyr^{157}) are involved in the stabilization of the transition state. In neprilysin, ECE and ACE families, only a histidine residue seems to have such a function. The aminopeptidase family seems to be more related to the astacin family in which only a tyrosine residue is involved in the stabilization of the transition state [34].

In the case of the mutant $D^{226}A$ a large decrease in specific activity of thermolysin is observed, due to a modification of both the k_{cat} and K_m values. In the structure of thermolysin this aspartate is involved in a hydrogen bond with His^{231} which stabilizes the transition state [24] and the modifications observed here in the kinetic parameters are less important than those obtained when His^{231} was mutated. The decrease in specific activity of the $D^{226}A$ mutant cannot be attributed to a direct effect of the mutation but rather to an indirect effect. In fact, the presence of an alanine at this position preventing the formation of the hydrogen bond with His^{231} probably leads to a move of this residue and a less efficient stabilization of the transition state by a local perturbation of the H bond network.

The mutant $D^{709}A$ in neprilysin also showed a large decrease in specific activity (300-fold) due only to a large decrease in the k_{cat} value. Curiously, this effect is significantly greater than that previously obtained when His^{711} in neprilysin was mutated in glutamine or phenylalanine [10] where the k_{cat} value was only decreased by a factor of 40. The effects obtained with these Asp→Ala mutations on the K_i values of the inhibitors are also very different in both enzymes. In thermolysin these effects are very low as compared to those obtain in neprilysin when Asp^{709} was mutated. From these data, it could be proposed that in neprilysin the main role of Asp^{709} is to stabilize the structure of the active site by preserving the right position of His^{711} as unique proton donor in the transition state. This could explain the large perturbation in neprilysin activity induced by the absence of Asp^{709}. In the case of thermolysin, it could be proposed that the perturbation of the hydrogen bond network associated with the $D^{226}A$ mutation could induce a slight reorganization of the active site in the vicinity of His^{231}. This perturbation is not highly detrimental for catalysis since the presence of Tyr^{157} could counterbalance the loss of efficiency of His^{231} in the stabilization of the transition state.

In conclusion, these results have again confirmed that the organization of the catalytic sites of thermolysin and neprilysin is very similar. However, although the overall mechanisms of action are similar, the mode of stabilization of the transition state differs between the two enzymes. These results also confirmed the importance of hydrogen bond networks in the active sites of both enzymes yet their importance and interdependence are not the same.

Acknowledgements: The authors would like to thank Prof. Bernard P. Roques for helpful discussions and critical reading of the manuscript.

References

[1] Rawlings, N.D. and Barrett, A.J. (1995) Methods Enzymol. 248, 183–228.
[2] Roques, B.P., Noble, F., Daugé, V., Fournié-Zaluski, M.C. and Beaumont, A. (1993) Pharmacol. Rev. 45, 87–146.
[3] Endo, S.J. (1962) J. Ferment. Technol. 40, 346–353.
[4] Matthews, B.W. (1988) Acc. Chem. Res. 21, 333–340.
[5] Devault, A., Lazure, C., Nault, C., Le Moual, H., Seidah, N., Chretien, M., Kahn, P., Powell, J., Mallet, J., Beaumont, A., Roques, B.P., Crine, P. and Boileau, G. (1987) EMBO J. 6, 1317–1322.
[6] Benchetrit, T., Bissery, V., Mornon, J.P., Devault, A., Crine, P. and Roques, B.P. (1988) Biochemistry 27, 592–596.
[7] Devault, A., Sales, V., Nault, C., Beaumont, A., Roques, B.P., Crine, P. and Boileau, G. (1988) FEBS Lett. 231, 54–58.
[8] Le Moual, H., Devault, A., Roques, B.P., Crine, P. and Boileau, G. (1991) J. Biol. Chem. 266, 15670–15674.
[9] Devault, A., Nault, C., Zollinger, M., Fournie-Zaluski, M.C., Roques, B.P., Crine, P. and Boileau, G. (1988) J. Biol. Chem. 263, 4033–4040.
[10] Dion, N., Le Moual, H., Crine, P. and Boileau, G. (1993) FEBS Lett. 318, 301–304.
[11] Le Moual, H., Dion, N., Roques, B.P., Crine, P. and Boileau, G. (1994) Eur. J. Biochem. 221, 475–480.
[12] Marie-Claire, C., Ruffet, E., Antonczac, S., Beaumont, A., O'Donohue, M.J., Roques, B.P. and Fournié-Zaluski, M.C. (1997) Biochemistry 36, 13938–13945.
[13] Roques, B.P., Fournié-Zaluski, M.C., Soroca, E., Lecomte, J.M., Malfroy, B., Llorens, C. and Schwartz, J.C. (1980) Nature 288, 286–287.
[14] Bouboutou, R., Waksman, G., Devin, J., Fournié-Zaluski, M.C. and Roques, B.P. (1984) Life Sci. 35, 1023–1030.
[15] Eijsink, V.G.H., Vriend, G., Van Der Burg, B., Venema, G. and Stulp, B.K. (1990) Protein Eng. 4, 99–104.
[16] O'Donohue, M.J., Roques, B.P. and Beaumont, A. (1994) Biochem. J. 300, 599–603.
[17] Bron, S. (1990) in: Molecular Biological Methods for *Bacillus* (Harwood, C.R. and Cutting, S.M., Eds.), Modern Microbiological Methods Series, New York.
[18] Nicoletti, V.G. and Condorelli, D.F. (1993) BioTechniques 14, 536.
[19] Sambrook, F., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[20] Benchetrit, T., Fournié-Zaluski, M.C. and Roques, B.P. (1987) Biochem. Biophys. Res. Commun. 147, 1034–1040.
[21] Llorens, C., Malfroy, B., Schwartz, J.C., Gacel, G., Roques, B.P., Roy, J., Morgat, J.L., Javoy-Agid, F. and Agid, Y. (1982) J. Neurochem. 39, 1081–1089.
[22] Marie-Claire, C., Roques, B.P. and Beaumont, A. (1998) J. Biol. Chem. 273, 5697–5701.
[23] Sansom, C.E., Hoang, V.M. and Turner, A.J. (1995) J. Cardiovasc. Pharmacol. 26, S75–S77.
[24] Beaumont, A., O'Donohue, M.J., Paredes, N., Rousselet, N., Assicot, M., Bohuon, C., Fournié-Zaluski, M.C. and Roques, B.P. (1995) J. Biol. Chem. 270, 16803–16808.
[25] Lee, S., Zambas, E.D., Marsh, W.L. and Redman, C.M. (1991) Proc. Natl. Acad. Sci. USA 88, 6353–6357.
[26] Mierau, I., Tan, P.S.T., Haandrikman, A.J., Kok, J., Leenhouts, K.J., Konings, W.N. and Venema, G. (1993) J. Bacteriol. 175, 2087–2096.
[27] Schmidt, M., Kröger, B., Jacob, E., Seulberger, H., Subkowski, T., Otter, R., Meyer, T., Schmalzing, G. and Hillen, H. (1994) FEBS Lett. 356, 238–243.
[28] Shimada, K., Takahashi, M. and Tanzawa, K. (1994) J. Biol. Chem. 269, 18275–18278.
[29] Emoto, N. and Yanagisawa, M. (1995) J. Biol. Chem. 270, 15262–15268.
[30] Lipman, M.L., Panda, D., Bennet, H.P.J., Henderson, J.E., Shane, E., Shen, Y., Goltzman, D. and Karaplis, A.C. (1998) J. Biol. Chem. 273, 13729–13737.
[31] Sen, I., Kasturi, S., Jabbar, M.A. and Sen, G.C. (1993) J. Biol. Chem. 268, 25748–25754.
[32] Chen, Y.N.P., Ehlers, M.R.W. and Riordan, J.F. (1992) Biochem. Biophys. Res. Commun. 184, 306–309.
[33] Vazeux, G., Iturrioz, X., Corvol, P. and Llorens-Cortes, C. (1997) Biochem. J. 327, 883–889.
[34] Stöckler, W., Gomis-Rüth, F.X., Bode, W. and Zwilling, R. (1993) FEBS Lett. 214, 215–231.