Evidence by Site-Directed Mutagenesis That Arginine 203 of Thermolysin and Arginine 717 of Neprilysin (Neutral Endopeptidase) Play Equivalent Critical Roles in Substrate Hydrolysis and Inhibitor Binding

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ABSTRACT: Neprilysin (neutral endopeptidase-24.11, EC 3.4.24.11) is a mammalian zinc-endopeptidase involved in the degradation of biologically active peptides. Although no atomic structure is available for this enzyme, site-directed mutagenesis studies have shown that its active site resembles closely that of the bacterial zinc-endopeptidase, thermolysin (EC 3.4.24.27). One active site residue of thermolysin, Arg-203, is involved in inhibitor binding by forming hydrogen bonds with the carbonyl group of a residue in the P_1' position and also participates in a hydrogen bond network involving Asp-170. Sequence alignment data shows that Arg-717 of neprilysin could play a similar role to Arg-203 of thermolysin. This was investigated by site-directed mutagenesis with Arg-203 of thermolysin and Arg-717 of neprilysin being replaced by methionine residues. This led, in both cases, to decreases in k_{cat}/K_m values, of 122-fold for neprilysin and 2300-fold for thermolysin, essentially due to changes in k_{cat}. The K_i values of several inhibitors were also increased for the mutated enzymes. In addition, the replacement of Asp-170 of thermolysin by Ala residue resulted in a decrease in k_{cat}/K_m of 220-fold. The results, coupled with a molecular modeling study, suggest that Arg-717 of neprilysin corresponds to Arg-203 of thermolysin and that in both enzymes a hydrogen bond network exists, involving His-142, Asp-170, and Arg-203 in thermolysin and His-583, Asp-650, and Arg-717 in neprilysin, which is crucial for hydrolytic activity.

Neprilysin (neutral endopeptidase-24.11, EC 3.4.24.11) is a mammalian, type II integral membrane protein expressed in many tissues and involved in the inactivation of a wide variety of signaling peptides such as enkephalins and atrial natriuretic factor [reviewed in Roques *et al*. (1993)]. Neprilysin (Devault *et al*., 1987; Letarte *et al*., 1988) belongs to the neprilysin family of zinc-endopeptidases (Rawlings & Barrett, 1995) which also includes the endothelin-converting enzymes (ECE) (Schmidt *et al*., 1994; Shimada *et al*., 1994; Emoto & Yanagisawa, 1995), the Kell blood group protein (Lee *et al*., 1991), and pepO from *Lactococcus lactis* (Mierau *et al.*, 1993). There is considerable interest in inhibitors of neprilysin as analgesics and antihypertensive agents (Roques & Beaumont, 1990; Roques *et al*., 1993) and more recently in inhibitors of ECE-1, also as potential antihypertensives (Turner & Murphy, 1996). However, as no member of the neprilysin family has been crystallized to date, a different strategy has been employed to obtain the active site models necessary to facilitate the design of potent and selective inhibitors.

Early structure−activity studies (Fournié-Zaluski *et al*., 1981; Hersh & Morihara, 1986) showed the similarities in the subsite specificity and structural organization of the active site of neprilysin and the bacterial zinc-metallopeptidase thermolysin (EC 3.4.24.27). Both enzymes cleave common substrates at the N-terminal side of hydrophobic residues and are inhibited by the same type of molecules, such as phosphoramidon (Fulcher *et al*., 1982) and thiorphan (Benchetrit *et al*., 1987) and have the same stereochemical dependence for optimal inhibition (Benchetrit *et al*., 1987; Roderick *et al*., 1989). Thermolysin, a thermostable neutral protease from *Bacillus thermoproteolyticus* Rokko (Endo, 1962), is a 316 amino acid protein which contains a catalytic zinc ion and four structural calcium ions. The enzyme has been crystallized in the presence of various inhibitors, including thiorphan and retrothiorphan (Roderick *et al*., 1989) and the study of the three dimensional structures of the complexes has allowed a general enzymatic mechanism to be proposed for zinc-metalloproteases [reviewed in Matthews (1988)]. The zinc atom in the active site is coordinated by three residues (His-142, His-146, and Glu-166) and a water molecule (Figure 1). It is assumed that during catalysis this water molecule, polarized under the influence of Glu-143 and the zinc ion, attacks the scissile bond. Other active site

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[1] ABBREVIATIONS: D170A-TLN, thermolysin in which Asp^{170} has been mutated to Ala; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; Phe-Ala, L-phenylalanyl-L-alanine; Phosphoramidon, N-(α-rhamnopyransoyl-(oxyhydroxyphosphinyl))-L-leucyl-l-tryptophan; R717M-neprilysin, neprilysin in which Arg^{717} has been mutated to Met; R203M-TLN, thermolysin in which Arg^{203} has been mutated to Met; Retrothiorphan, 3-((1-(*R*,*S*)-(mercaptomethyl)-2-oxo-3-phenylethyl)amino)-3-oxopropanoic acid; rmsd, root mean square distance; thiorphan, *N*-(2-(*R*,*S*)-mercaptomethyl)-1-oxo-3-phenylpropyl)glycine.

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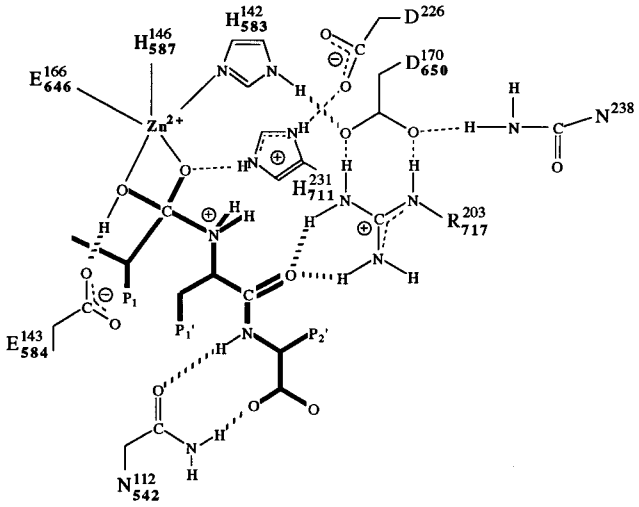


FIGURE 1: Schematic representation of the network of hydrogen bonds of the active site of thermolysin. This model show the apparent network of hydrogen bonds (broken lines) between the residues involved in catalysis in the active site. The corresponding residues in neprilysin are given in bold.

residues include His-231, which stabilizes the transition state, Asn-112 and Arg-203, involved in substrate binding, and Asp-170, involved in a hydrogen bond network (Holmes & Matthews, 1982) (Figure 1). A comparison of the sequences of neprilysin and thermolysin by hydrophobic cluster analysis (Benchetrit *et al.*, 1988) suggested similarities in the active sites of the two enzymes, and this has since been confirmed by site-directed mutagenesis for the three zinc-binding ligands, His-583, His-587, and Glu-646 (Devault *et al*., 1988b; Le Moual *et al*., 1991), and the catalytic Glu-584 (Devault *et al*., 1988a). In addition Asn-542 (Dion *et al*., 1995), Asp-650 (Le Moual *et al*., 1994), and His-711 (Bateman *et al*., 1990; Kim *et al*., 1992; Dion *et al*., 1993) have been proposed to be the counterparts of Asn-112, Asp-170, and His-231 of thermolysin. These residues are also conserved in other members of the neprilysin family (Figure 2) and site-directed mutagenesis studies have shown the equivalent roles of some of these residues in ECE-1 (Shimada *et al*., 1996) and pepO (Lian *et al*., 1996).

Arg-203 of thermolysin participates in ligand binding by forming hydrogen bonds with the carbonyl group of a P_1' residue (Matthews, 1988), and it has been proposed previously that Arg-747 could be the equivalent residue in neprilysin (Benchetrit *et al*., 1988; Beaumont *et al*., 1991). However, Arg-747 is not conserved in the other members of the neprilysin family, whose sequences have since become available, and an alignment of these sequences shows that only one residue, corresponding to Arg-717 of neprilysin could correspond to Arg-203 of thermolysin (Figure 2). Arg-717 has therefore been mutated to Met and the consequences of this mutation on substrate hydrolysis and inhibitor binding have been investigated. A parallel mutation of Arg-203 was carried out in thermolysin to enable a direct comparison to be made. Moreover, as Asp-170 of thermolysin is involved in interactions with Arg-203, this residue was mutated to Ala and the results compared to those previously described for the corresponding, D650A mutation of neprilysin (Le Moual *et al*., 1994). The results suggest that Arg-717 of neprilysin is the counterpart of Arg-203 of thermolysin and that in both enzymes the arginine residue is involved in a crucial hydrogen bond network involving the zinc ions and His-142, Asp-170, and Arg-203 in thermolysin and His-583, Asp-650, and Arg-717 in neprilysin. It appears that, through their involvement in these networks, Arg-203 of thermolysin and Arg-717 of neprilysin can play a role in the catalytic processes of their respective enzymes.

MATERIALS & METHODS

Materials. Restriction and modifying enzymes, bacterial culture media, and the Transformer Site-Directed Mutagenesis kit were, respectively, from New England Biolabs, Bio101, and Clontech products and obtained from Ozyme (France). The Sequenase enzyme version 2.0 (United States Biochemical) and [α-^{33}P]dATP were purchased from Amersham (France). Cell culture products were from Life Technologies (France). pSVP4-neprilysin plasmid was kindly provided by Dr. G. Boileau (Canada) and Mabs Alb1 and T1 were generous gifts from Dr. C. Boucheix and Dr. C. Bohuon (Villejuif, France), respectively. pRSET(B) and pcDNA3 are, respectively, procaryotic and eukaryotic expression vectors available from Invitrogen Corp. First (U.K.). Oligonucleotides were synthesised by Genosys (U.K.). 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 and the Phenyl Superose column were purchased from Pharmacia (France) and bestatin, captopril, and phosphoramidon from Sigma (France). The other inhibitors used, thiorphan (Roques *et al*., 1980), retrothiorphan (Fournié-Zaluski *et al*., 1986), and Phe(ΨCH$_2$NH)-Ala were prepared in the laboratory following classical procedures.

Vector Construction and Site-Directed Mutagenesis. The *Bacillus* strain used was *Bacillus Subtilis* DB117, lacking neutral protease activity (Eijsink *et al*., 1990). The pTLN2 vector encoding the complete sequence of preprothermolysin has been previously described (O'Donohue *et al*., 1994). pTLN2 was digested with endonucleases *Bam*HI and *Eco*RI to release the sequence of mature thermolysin. This fragment was then subcloned in the pRSET(B) vector. The resulting plasmid (pTLN-Mat3) encoding the sequence of the mature enzyme was used for the double strand mutagenesis in *Escherichia Coli*. The mutated fragment *Eco*R/ *Mlu*1 was then subcloned in pTLN2 encoding the full length of the enzyme to express the enzymes in *B. subtilis* DB117 as previously described (O'Donohue *et al*., 1994). For plasmid transformation, *B. subtilis* protoplasts were prepared as described by Bron (1990), and a modified polyethylene glycol precipitation method was used for large-scale plasmid preparation (Maniatis *et al*., 1989). The plasmids pcDNA3 and pSVP4-neprilysin, encoding the full-length human neprilysin (Letarte *et al*., 1988) were used to create a new eukaryotic expression vector. pSVP4-neprilysin was digested with endonuclease *Mlu*1 to release a 2960 bp DNA fragment encoding the full-length enzyme. The cDNA fragment was then subcloned in the *Eco*RV restriction site of pcDNA3 vector. The orientation was determined by restriction nuclease mapping and sequencing. Double-strand mutagenesis was carried out using the Transformer Site-Directed Mutagenesis kit following the manufacturer's instructions. Oligonucleotides were designed to replace Arg-203 and Asp-

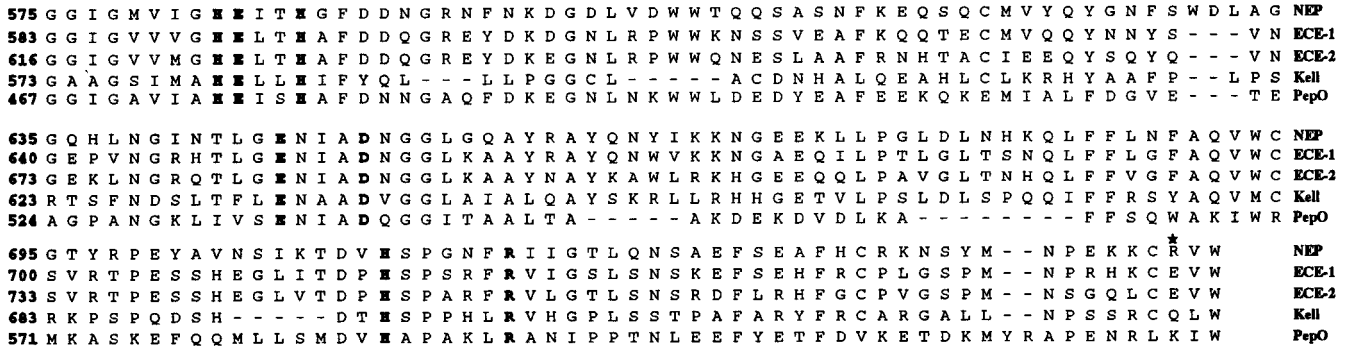


FIGURE 2: Alignment of the C-terminal sequences of NEP (neprilysin) (Letarte *et al.*, 1988), ECE-1 (Shimada *et al.*, 1994), ECE-2 (Emoto & Yanagisawa, 1995), Kell (Lee *et al.*, 1991), and PepO (Mierau *et al.*, 1993). Conserved residues, shown by site-directed mutagenesis to be active site residues of NEP (neprilysin) are in bold: His-583, His-587, Glu-646 (Devault *et al.*, 1988b; Le Moual *et al.*, 1991), Glu-584 (Devault *et al.*, 1988a), Asp-650 (Dion *et al.*, 1995), and His-711 (Kim *et al.*, 1992). The conserved Arg is also in bold and Arg-747 of neprilysin is marked with ★.

170 in thermolysin to Met and Ala, respectively, and Arg-717 in neprilysin to Met. The authenticity of each mutation was confirmed by sequencing the complete coding sequence.

Expression and Purification of Wild-Type and Mutated Thermolysins. B. subtilis DB117 cells harboring the plasmid pTLN2 were grown in LB broth containing 5 mM $CaCl_2$ and 5 μg/mL chloramphenicol at 37 °C with shaking 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 previously described (O'Donohue *et al*., 1994), except that the enzymes were eluted from the affinity column in 100 mM Tris-HCl, pH 9.0, containing 5 mM $CaCl_2$. The eluate was immediately neutralized, made up to 40% saturated $(NH_2)_2SO_4$ and applied to a 1 mL column of Phenyl Superose, equilibrated in 50 mM Tris-HCl, pH 7.0, and 40% saturated $(NH_2)_2SO_4$, and the enzymes were eluted at 0.5 mL/min as a sharp peak, at the end of a gradient of 40 to 0% $(NH_2)_2SO_4$ in 40 min. The latter column served to concentrate the enzymes and also eliminated a minor band of 38 kDa observed on SDS−PAGE after elution of R203M-TLN enzyme from the affinity column.

Cell Culture and Transfection of Wild-Type and Mutated Neprilysins. COS-7 cells were grown in DMEM supplemented with 10% foetal calf serum and 50 μg/mL gentamicin at 37 °C. Cells were seeded into 10 cm Petri dishes, at a density of 10^6 cells /dish and 24 h later were transfected with a pcDNA3 vector encoding wild-type or mutant neprilysin, using a DEAE-dextran-chloroquine method (Maniatis *et al*., 1989). Cells were solubilized 48 h after transfection 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 20000*g* for 15 min, and the supernatant was used for enzymatic assays. Western blot analysis was performed to detect neprilysin expression in cell extracts.

Western Blotting. Proteins were subjected to 8 or 12% sodium dodecyl sulfate−polyacrylamide gels electrophoresis (SDS−PAGE) and electroblotted onto nitrocellulose filters. The blots were sequentially incubated with Superblock blocking buffer (Pierce), mAb Alb1 raised against human neprilysin or mAb T1 raised against thermolysin and finally with an anti-mouse Ig, horseradish peroxidase linked antibody from sheep (Amersham Corp.). The peroxidase activity was revealed with a chemioluminescent detection kit from Amersham Corp.

Enzyme Assays. Thermolysin activity and K_i values of inhibitors were assayed, as previously described (Benchetrit *et al*., 1987), at 37 °C in a total volume of 100 μL of 50 mM HEPES, pH 6.8, containing 5 mM $CaCl_2$, with 20 nM [^{3}H]leucine enkephalin as a substrate. The reaction was stopped by adding 10 μL of 0.5 M HCl, and the metabolite [^{3}H]tyrosyl-glycyl-glycine was separated using Porapaq Q beads from Waters (France). K_m and k_{cat} values were determined by the isotopic dilution method. Calculations were carried out using the program ENZFITTER (Biosoft).

Neprilysin activity and K_i values of inhibitors were measured using 20 nM [^{3}H]D-Ala^2-leucine enkephalin as substrate (Llorens *et al*., 1982). Assays were carried out in 100 μL of Tris-HCl, pH 7.4, at 37 °C, and the reaction was stopped by adding 10 μL of 0.5 M HCl and the metabolite separated on Poropaq Q beads. Due to the use of crude neprilysin preparations, incubations also contained 1 μM captopril (angiotensin-converting-enzyme inhibitor) and 10 μM bestatin (aminopeptidase inhibitor). Indeed we have previously shown that aminopeptidases are able to generate small amount of Tyr by cleavage of the Tyr-D-Ala bond (Llorens *et al*., 1982). K_m values were determined by the isotopic dilution method. Calculations were carried out using the program ENZFITTER (Biosoft). In order to calculate the k_{cat} values, neprilysin levels were determined by densitometry after a dot blot, using a monoclonal antibody raised against the human enzyme, whose epitope is away from the active site. Pure recombinant human neprilysin (generously provided by Khepri Pharmaceutical Inc.) was used to construct a calibration curve. Neprilysin levels measured by this method correlate well with those determined by other methods such as active site titration with a radioactive inhibitor (not shown). IC_{50} values were considered to be equal to K_i values as the concentration of the substrate used was less than its K_m for the enzyme. In all cases, the reactions were stopped when substrate degradation was $\leq$10%.

Structural Calculations. The structure of thermolysin crystallized with thiorphan was used for calculations (Roderick *et al*., 1989). The computations were performed using the Discover program developed by Biosym Technologies, Inc., (San Diego) containing the Amber force field (Weiner *et al*., 1986) parametrized for the zinc atom from *ab initio* calculations. The structure of the mutated enzyme was obtained by replacing Arg-203 for Met, using the Biopolymer unit of the Biosym package. Final structures were obtained

by minimization procedures with the molecular dynamic simulations followed by minimization in the case of the thermolysin mutant. A leap-frog algorithm with a time step of 1 fs was used during the molecular dynamic simulations. The most common dynamic protocol used was based on a 2 ps equilibration stage followed by a 10 ps conformation production stage performed at 300 K, applied with or without constraints. A conformation was recorded every 100 fs. The 100 conformations obtained by this protocol were then energetically and structurally analyzed and the four best were minimized. Minimizations were performed using either steepest descent or conjugate gradients methods without any constraints.

RESULTS

Mutagenesis of Thermolysin and Neprilysin cDNA and Expression of the Mutated Enzymes. The plasmids pTLN2, pTLN-R203M, and pTLN-D170A encoding wild-type and mutated thermolysins were transformed in *B. subtilis* DB117 protoplasts. Wild-type and mutant thermolysins were purified from the culture supernatant using a Gly-D-Phe affinity column followed by Phenyl Superose chromatography, with yields of approximately 4 mg/L for wild-type enzyme and 300 μg/L for R203M-TLN and D170A-TLN. Thermolysin is synthesized as a preproenzyme (O'Donohue *et al*., 1994), and the lower yields of the mutant enzymes are probably due to a decrease in the autocatalytic processing of the prosequence provoked by the active site mutation. In line with this, increased intracellular levels of the proenzyme forms were found in *Bacilli* expressing mutant thermolysins, as compared to *Bacilli* expressing the wild-type enzyme (not shown), and mutations which reduce the catalytic activity of other thermolysin-like bacterial enzymes have previously been shown to decrease the yields of mature enzyme, (Toma *et al*., 1989; McIver *et al*., 1991; Kawamoto *et al*., 1993; Beaumont *et al*., 1995). Western blot analysis, with mAb T1 showed, for the three mature enzymes, an apparent molecular mass of 36 kDa (Figure 3A).

For neprilysin, the plasmids pcDNA-neprilysin and pcDNA-R717M carrying the mutation were transiently expressed in COS cells, and the presence of the mutated and wild-type enzymes in cell extracts was determined by Western blot analysis. Correct glycosylation of the enzymes was verified by endoglycosidase H and N-glycosidase F digestion (data not shown). Wild-type and mutated enzymes had similar apparent molecular masses (98 kDa) (Figure 3B) and expression levels, indicating that the mutation did not interfere with the production of neprilysin. For both thermolysin and neprilysin, the wild-type and mutated enzyme preparations were stable for up to 4 weeks at 4 °C as well as under the assay conditions used.

Enzymatic Properties of Wild-Type and Mutated Enzymes. Leucine enkephalin and its D-Ala^2-Leu-enkephalin analogue are equally good substrates for neprilysin (Llorens *et al*., 1982); however, only the former peptide is hydrolyzed by thermolysin (Hersh & Morihara, 1986). D-Ala^2-Leu-enkephalin was therefore used for the mammalian enzyme, due to its better resistance to aminopeptidase activity, and leucine enkephalin for thermolysin. When the K_m and k_{cat} values for the degradation of leucine enkephalin were measured for wild-type and R203M-TLN (Table 1), the K_m value was found to be very similar for both enzymes.

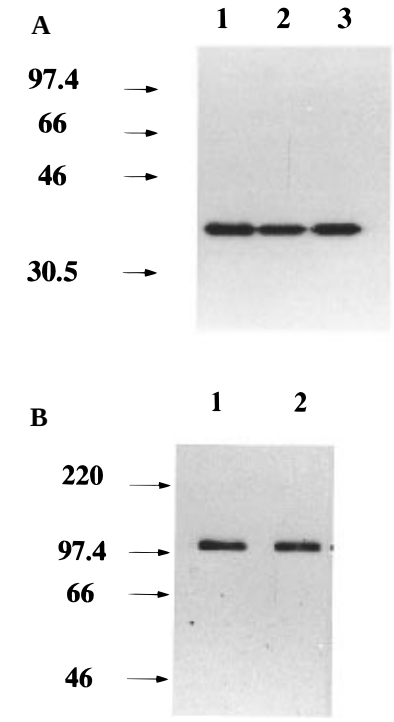


FIGURE 3: Analysis of wild-type and mutated enzymes by western blots as described in Materials and Methods. (A) Lane 1, wild-type thermolysin; lane 2, R203M, lane 3, D170A. (B) Lane 1, wild-type neprilysin; lane 2, R717M. Position of molecular mass markers (kDa) are indicated on the left side.

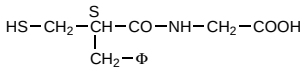
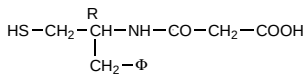
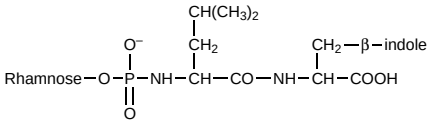
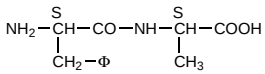
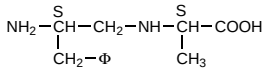
Table 1: Kinetic Constants for the Hydrolysis of Leucine Enkephalin by Wild-Type, R203M, and D170A-TLN and for the Hydrolysis of D-Ala^2-Leucine Enkephalin by Wild-Type and R717M-NEP[a]

enzymes	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
		Thermolysin	
wild-type	200 ± 25	9330 ± 750	46.6
R203M	240 ± 1	5 ± 0.1	0.02
D170A	480 ± 26	100 ± 2	0.21
		Neprilysin	
wild-type	45 ± 9	1600 ± 265	35.5
R717M	40.4 ± 6	12 ± 3	0.29

[a] Reactions were carried out as described in Materials and Methods. Data are the mean ± SEM from at least three independent determinations.

However, the k_{cat} value of R203M-TLN was nearly 2000-fold lower than that of the wild-type enzyme. With neprilysin, the K_m value of the substrate D-Ala^2-leucine enkephalin for R717M-NEP was also very similar to that for wild-type enzyme, while the k_{cat} value was 133-fold lower (Table 1). Thus, for both thermolysin and neprilysin, the arginine to methionine mutation provoked a reduction in the specificity factor k_{cat}/K_m, which was essentially due to changes in k_{cat}, of 2300-fold for the bacterial enzyme and 122-fold for the mammalian enzyme. For D170A-TLN, the K_m for the degradation of leucine enkephalin was very similar to that of the wild-type enzyme while the k_{cat} of the mutant enzyme was 93-fold lower than that of the wild-type thermolysin. The specificity factor k_{cat}/K_m was thus reduced 220-fold as compared to the wild-type enzyme. It should be noted that, as in all three cases, the K_m values of the substrates for the mutant enzymes were only slightly increased; as compared to their controls, it seems unlikely that the mutations provoked any major destabilization of the active sites.

Table 2: K_I Values of Inhibitors for Wild-Type and Mutated Thermolysin and Neprilysin[a]

inhibitors	neprilysin, K_I (μM)		thermolysin, K_I (μM)		
	wild-type	R717M	wild-type	R203M	D170A
 Thiorphan	0.0024 ± 0.0008	29 ± 6	1.6 ± 0.2	160 ± 20	3.3 ± 0.2
 Retrothiorphan	0.0039 ± 0.0003	5.2 ± 1.1	0.8 ± 0.1	160 ± 50	nd
 Phosphoramidon	0.0008 ± 0.0002	>1000	0.06 ± 0.006	4.7 ± 0.5	*b*
 Phe-Ala	5.5 ± 0.8	2300 ± 100	nd	nd	nd
 Phe(Ψ CH_2NH)Ala	2800 ± 600	10000 ± 4000	nd	nd	nd

[a] Data are the mean ± SEM from at least three independent determinations. [b] Biphasic curves.

The arginine mutations also caused a shift in pH dependence, the pH optima for R203M-TLN and R717M-NEP being 5.5 and 5.75, respectively, compared to pH 6.8 for the wild-type enzymes. However, the k_{cat}/K_m ratios for wild-type/mutant enzymes were not significantly changed when measured at the more acidic pH.

Inhibitor Binding to Wild-Type and Mutated Enzymes. A decrease in the inhibitory potencies of the thiol inhibitors, thiorphan (100-fold) and retrothiorphan (200-fold) and the phosphoramidate inhibitor phosphoramidon (78-fold) were observed for R203M-TLN (Table 2). With R717M-NEP, there were even larger decreases in the inhibitory activities of thiorphan (12000-fold) and retrothiorphan (1300-fold), and the K_i value of phosphoramidon was increased by over 6 orders of magnitude (Table 2). The dipeptide Phe-Ala had a 418-fold higher K_i value, for R717M-NEP, than for the wild-type enzyme. However, when the carbonyl between the P_1' and P_2' residues was replaced with a CH_2 in Phe(ΨCH_2NH)Ala, only a slight difference was observed (3.5-fold). The K_i of this latter inhibitor was too high to be measured for wild-type and R203M-TLN.

In the case of D170A-TLN, the K_i of thiorphan was similar to that of the wild-type enzyme (Table 2), while with phosphoramidon the inhibition curves obtained were biphasic and could be interpreted as a two-site model with K_i values of 2.5 μM and >1 mM. The reasons for these biphasic curves are unknown, although interestingly similar results have been reported for phosphoramidon inhibition of ECE-1 (Balwierczak *et al.*, 1995).

Molecular Modeling Studies. The molecular modeling studies performed with thermolysin cocristallized with *S*-thiorphan (Roderick *et al.*, 1989) showed that the mutation of Arg-203 to Met leads to a reorganisation of the amino acids lying in the active site of thermolysin (the rmsd for the best superposition of the active site amino acids, except for residue 203, was around 1.1 Å when using all atoms, except hydrogen, and around 0.80 Å when using backbone atoms only). This structural change is mainly due to a rearrangement of a hydrogen bond network in the Met-203 mutant (Figure 4). Crystallographic and minimized structures of the wild-type enzyme have shown that Asp-170 plays an important role in the positioning of Arg-203, through the formation of two hydrogen bonds (1.83 and 1.97 Å) between its carbonyl oxygen atoms and the guanidinium group of Arg-203 (Figure 4). This interaction cannot occur with Met-203, the side chain of which moves closer to two hydrophobic residues, Ile-188 and Leu-202, in the mutant structure. The loss of hydrogen bonds between Asp-170 and Met-203 also leads to a repositioning of the acidic residue, reducing its interaction with the δ NH of His-142, one of the zinc-binding ligands (the distance of 1.95 Å in the wild-type enzymes becomes 3.8 Å in the mutated enzyme). Conversely, the hydrogen bond with the amine group of Asn-238 is strengthened (2.15 Å in the wild-type enzyme vs 1.70 Å in the mutated enzyme). Concomitant with the positioning of Met-203, there is a movement of His-231 toward the oxygen atom of the amide carbonyl group of the P_1' residue allowing a hydrogen bond to be formed between the ϵ NH of His-231 and the carbonyl group (3.40 Å in the wild-type enzyme vs 2.5 Å in the mutated enzyme). Apart from His-231, there are few changes in the positions of the other active site amino acids. The other hydrogen bonds, involving Asn-112 for example, which stabilize the binding of the substrate or the inhibitor are unchanged. Molecular dynamic simulations followed by energy minimization procedures showed that no significant energy change was induced by the mutation.

DISCUSSION

Many of the residues established as being important at the active site of neprilysin have their equivalents in thermolysin and are also conserved in the other members of the neprilysin family (Figure 2), and it might be expected

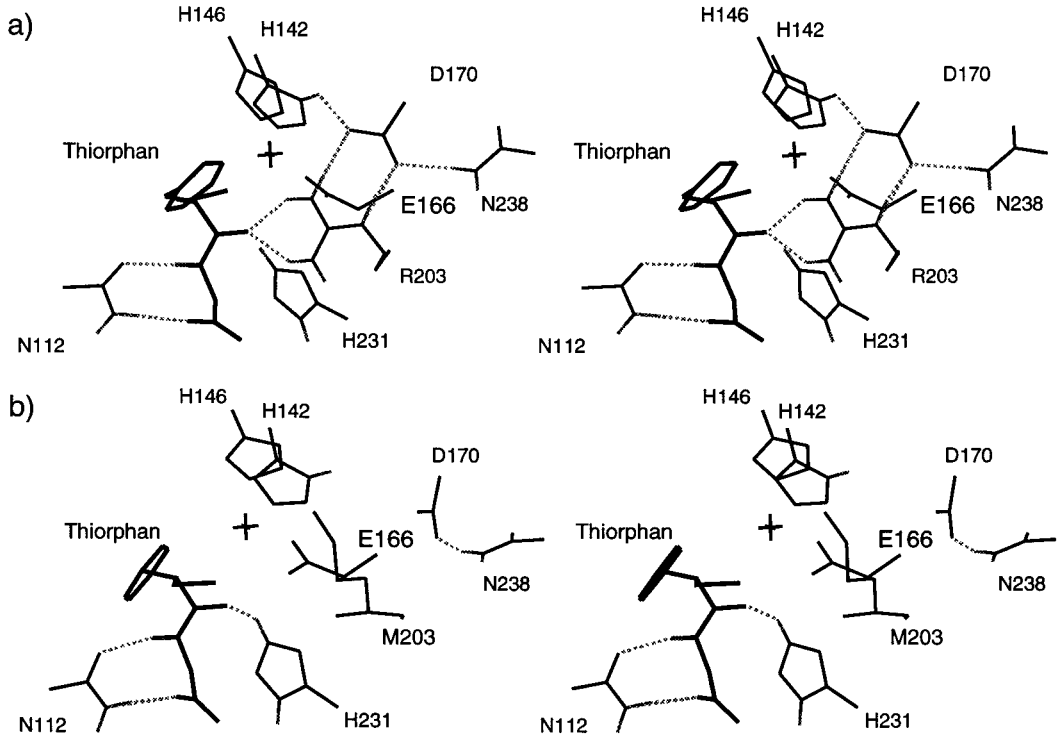


FIGURE 4: Stereo views of the thiol inhibitor thiorphan bound to thermolysin active site. His-231 and Arg-203 are in bold. (a) Wild-type thermolysin−thiorphan complex. (b) R203M-TLN−thiorphan complex. The active site zinc atom is represented by X.

that an arginine residue which participates in substrate binding in the interior of the active site and is not involved in specificity would also be conserved. This is not the case for Arg-747 of neprilysin (Figure 2), originally proposed as being the equivalent of Arg-203 of thermolysin (Beaumont *et al*., 1991), and the sequence alignment showed that only Arg-717 of neprilysin is conserved in the neprilysins known to date. The cloning of thermolysin (O'Donohue *et al*., 1994) has also enabled site-directed mutagenesis to be applied to this enzyme, allowing parallel mutations to be carried out in the mammalian and bacterial peptidases, and a direct comparison to be made of the effects of changing putative equivalent residues. Both Arg-717 of neprilysin and Arg-203 of thermolysin were therefore mutated to Met.

These mutations provoked, for both enzymes, shifts in optimum pH toward the acidic region, and large decreases in k_{cat}/K_m values and increases in the K_i values of several inhibitors (Table 1 and 2). In contrast, when Arg-747 of neprilysin was mutated to Met, only a comparatively slight, 3-fold decrease in k_{cat}/K_m values was observed, mostly due to changes in K_m, and the K_i value of thiorphan was only increased 4-fold (Beaumont *et al*., 1991). In line with this, when the glutamate of ECE-1, which aligns with Arg-747, was mutated, the changes observed were also small (Shimada *et al*., 1996). The results therefore indicate that Arg-717 and not Arg-747 of neprilysin is the equivalent of Arg-203 of thermolysin. The slight changes in substrate and inhibitor binding provoked by the R747M mutation might result from a proximity of Arg-747 to the active site or from transconformational changes, possibly affecting the disulphide bond formed by the adjacent residue Cys-746. However, further structural and modeling studies will be required to resolve the exact role and positioning of this residue.

As well as establishing Arg-717 as an active site residue of neprilysin, the results obtained give interesting insights into the roles of Arg-203 and Arg-717 in their respective enzymes. In the model of inhibitor binding, deduced from numerous crystallographic studies, Arg-203 stabilizes and probably ensures a *bona fide* conformation of the bound inhibitor, through the formation of two hydrogen bonds between its guanidinium group and the oxygen of the carbonyl of the P_1' residue, and it has also been proposed to stabilize substrate binding, without participating in the catalytic process (Matthews, 1988). It might therefore have been expected that mutating the corresponding arginines in thermolysin and neprilysin would affect K_m values to a greater extent than k_{cat} values, as occurred when two residues thought to be involved in ground state binding in neprilysin, Arg-102 (Bateman *et al*., 1989; Beaumont *et al*., 1992) and Asn-542 (Dion *et al*., 1995) were mutated. In contrast, when the histidine residues of a thermolysin-like enzyme (Beaumont *et al*., 1995) and neprilysin (Dion *et al*., 1993), proposed to participate in transition state stabilization, were mutated, the resulting changes in catalytic activity were mostly due to k_{cat}. However, little difference in the K_m values of the substrates was observed for R203M-TLN and R717M-NEP and their wild-type controls, while there were large reductions in the k_{cat} values of the mutated enzymes, of nearly 2000-fold for R203M-TLN, suggesting the involvement of the arginines in the catalytic process. In addition the thermolysin mutation produced similar reductions (100−200-fold) in the affinities of thiorphan and retrothiorphan, inhibitors considered to be collected product analogues (Roderick *et al*., 1989) and phosphoramidon, considered to be a transition state inhibitor (Tronrud *et al*., 1986). The loss of two strong hydrogen bonds might also be expected to decrease the binding affinities for the three inhibitors even more than observed, suggesting that compensatory stabilizing interaction(s) occur in the active site of R203M-TLN.

To try to explain the results obtained, a molecular modeling study was undertaken using the crystallographic data of the complex between thiorphan and thermolysin

(Roderick *et al*., 1989). This showed that the main consequence of the Arg-203 to Met mutation is a change in the positioning of His-231, the NH group of which is at a distance from the oxygen of the P_1' carbonyl of thiorphan, compatible with the formation of an hydrogen bond (Figure 4). This interaction, facilitated by a movement of the Met side chain toward a more hydrophobic environment, could compensate partially for the loss of the two hydrogen bonds formed by Arg-203 but reduces the ability of His-231 to participate in transition state stabilization, thus leading to a reduction in k_{cat}. A change in the environment of His-231 could also explain the changes in the optimum pH of the mutated enzymes, as the ionization state of this residue is thought to be responsible for the pH dependency of thermolysin at basic pH (Kunugi *et al*., 1982; Izquierdo-Martin & Stein, 1992). It should be noted that a different mechanism of action has been recently proposed for thermolysin, in which His-231 acts as the general base in catalysis (Mock & Stanford.1996; Mock & Aksamawti, 1994). In this case, a change in the position of His-231 would also be expected to reduce k_{cat} values.

Although the effects of mutating Arg-203 and Arg-717 in thermolysin and neprilysin, respectively, were similar overall, there were also interesting differences. The R203M mutation in thermolysin reduced the affinities of the inhibitors tested between 100- and 200-fold. However, for R717M-NEP, the K_i values of thiorphan and retrothiorphan increased 12000-fold and 1300-fold, respectively, and that of phosphoramidon was increased by over 6 orders of magnitude. In contrast, the decrease in k_{cat}/K_m resulting from the Arg-717 to Met mutation was only 122-fold as compared to the 2300-fold decrease observed for the corresponding replacement R203M in thermolysin. Taken together, these findings seem to indicate that the reorganization of the active site, leading in thermolysin to the formation of an hydrogen bond between His-231 and the carbonyl of a P_1' residue and the loss of its transition state stabilization, is probably more difficult in the mammalian enzyme, accounting for the comparatively larger loss in inhibitor affinity and smaller loss in k_{cat}. The structural differences between the active sites of thermolysin and neprilysin, illustrated by this site-directed mutagenesis study, remain to be determined by further molecular modeling studies.

In addition to substrate binding, Arg-203 of thermolysin also participates in a hydrogen bond network involving Zn^{2+}, His-142, and Asp-170, where His-142 is one of the three zinc-binding ligands (Figure 1). The importance of this network is shown here by the D170A-TLN mutation, which resulted in a 93-fold decrease in k_{cat}, with a slight, 2.4-fold, increase in K_m. Disruption of the network, combined with a change in the position of His-231 might therefore explain the large decrease in k_{cat} seen with the R203M mutant enzyme. For neprilysin, it has previously been shown that mutating Asp-650, the equivalent of Asp-170, also leads to a large, 500-fold loss of activity (Le Moual *et al*., 1994), and a similar hydrogen bond network involving Zn^{2+}, His-583, Asp-650, and Arg-717 can be proposed for this enzyme.

In conclusion, Arg-717 of neprilysin has been identified as being the equivalent of Arg-203 in thermolysin, and the similar organization of the active sites of the mammalian and bacterial enzymes has again been confirmed with, in particular, the participation of charged and polar residues in hydrogen bond networks which are of fundamental importance for the structure and stability of their respective active sites and for enzyme functioning.

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