

Characterisation of neprilysin (EC 3.4.24.11) S₂' subsite

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Abstract Neprilysin is a neutral peptidase that cleaves small peptide substrates on the amino-side of hydrophobic amino acid residues. In the present study, we have used inhibition of non-mutated and mutated enzymes with dipeptide inhibitors and hydrolysis of the substrate [Leu⁵, Arg⁶]enkephalin in order to evaluate the contribution of the S₂' subsite to substrate and inhibitor binding. Our results suggest that (1) Arg-102 and Asn-542 provide major contributions to the interaction of the enzyme with the P₂' residue of the substrate, (2) the S₂' subsite is vast and can accommodate bulky side chains, and (3) Arg-102 restricts access to the S₂' subsite to some side chains such as arginine.

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1. Introduction

Neprilysin (EC 3.4.24.11, NEP, neutral endopeptidase, CD10, CALLA) is a member of the gluzincin subfamily of zinc metalloproteases/peptidases [1]. This group of enzymes is characterized by the HEXXH active site consensus sequence where the histidine residues are two of the zinc ligands and the glutamate is involved in the catalytic mechanism. In gluzincins, a glutamic acid residue is the third zinc ligand. The primary structure of NEP has been deduced from cDNA sequences for the human, rabbit, rat and mouse enzymes, and show a high degree of similarity between species [2–5]. NEP is a 94-kDa enzyme that is anchored in the plasma membrane with a type II topology. Rabbit NEP consists of a short N-terminal cytoplasmic domain of 27 residues, a 23-residue membrane spanning region, and a large ectodomain of 699 residues. The enzyme also has 5 glycosylation sites and its tertiary structure is stabilized by 6 disulfide bridges. NEP is distributed in many tissues including kidney and intestinal microvilli, the central nervous system, and the immune system. It is involved in the extracellular catabolism of short bioactive peptides such as the enkephalins and the atrial natriuretic peptide. According to their antinociceptive and anti-hypertensive properties, NEP inhibitors are of great interest [6].

In peptidases, enzyme-substrate specificity is determined by the formation of interactions between enzyme subsites and specific residues of the substrate (residues of the substrate positioned in N terminus of the cleavage site are referred to

as P and interact with the S subsites of the enzyme whereas residues on the C-terminal side of the cleavage site are named P' and interact with the S' subsites of the peptidase; Fig. 1). In the case of NEP, these interactions have been studied essentially with natural and synthetic peptides or inhibitors [7–11]. It has been shown that NEP displays a preference for a hydrophobic residue in the P₁' position and for a substrate containing a free COOH-terminal carboxylate. The S₂' subsite is also important for the stabilisation of enzyme/substrate interactions. Indeed, mutagenesis studies have indicated that NEP Arg-102 forms a salt bridge or a hydrogen bond with the carboxyl/carbonyl group of the P₂' residue, and NEP Asn-542 establishes a hydrogen bond with the amino side of the same residue [12–14]. Furthermore, using synthetic substrates Quay et al. [11] suggested that the S₂' subsite of NEP prefers hydrophobic lateral chains. However, this subsite is known to accept charged residues such as arginine in bradykinin. To further investigate this NEP subsite preference, we have created mutant enzymes carrying the mutations R102M and/or N542G. Since these two NEP residues were shown to interact with the peptide backbone around the P₂' residue of the substrate, it was hypothesized that in the mutated enzymes interaction in the S₂' subsite would depend mostly on the nature of the P₂' lateral chain.

In this report we have used inhibition of single and double mutated enzymes with dipeptides and cleavage of [Leu⁵, Arg⁶]enkephalin to evaluate the interaction of NEP with the substrate P₂' residue. Our results suggest that (1) Arg-102 and Asn-542 provide important contributions to the interaction with the P₂' residue of the substrate, (2) the S₂' subsite is vast and can accommodate bulky side chains, and (3) Arg-102 restricts access to the S₂' subsite to some side chains such as arginine.

2. Materials and methods

2.1. Site-directed mutagenesis, vector construction and transfection of COS-1 cells

All DNA manipulations were performed using described procedures [15]. Oligonucleotide-directed mutagenesis was achieved according to the method described by Taylor et al. [16], with an M13 subclone containing the proper fragment of NEP cDNA. Recombinant M13-NEP phages carrying the mutations were screened directly by DNA sequencing [17]. A DNA fragment containing the mutated region was isolated from the replicating form of M13-NEP recombinant phage and substituted for the equivalent non-mutated fragment in pSVsec-NEP, a previously described NEP expression vector [18]. The presence of the mutations in the expression vectors was confirmed by sequencing the coding regions of the NEP cDNA by the chain-termination method for double-stranded templates using T7 DNA polymerase [19].

Non-mutated and mutated enzymes were produced by transient expression in COS-1 cells [20] using the standard calcium-phosphate coprecipitation procedure [21] as previously described [22]. The day following transfection, the serum containing medium was changed for

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Abbreviations: CALLA, common acute lymphoblastic leukaemia antigen; MES, 2-(morpholino)ethanesulfonic acid; NEP, neprilysin

'synthetic medium' (modified from Murakami et al. [23]) and described by Lemay et al. [18]. After 16 h of incubation, this medium was collected, filtrated through a 0.45 µm Millex membrane and concentrated (5-fold) on Centricon-30 (Amicon Canada).

2.2. Enzyme assays and immunoblotting

The enzymatic activity was determined as described previously [14] using 50 nM of the tritiated substrate [tyrosyl-(3,5 ³H)](D-Ala²)-Leu⁵-enkephalin (50 Ci/mmol) purchased from Research Product International Inc. The k_m values were determined by the isotopic dilution method. Calculations were done using the program ENZFITTER. In order to calculate the k_{cat} values, the amounts of enzyme were quantified by immunoblot analysis using a purified soluble form of rabbit NEP as standard [24]. Immunoblotting was performed as previously described using monoclonal antibody 18B5 [14]. The inhibitory effects of dipeptides (Sigma Chemical Co.) were determined by serial dilution of the inhibitor in enzymatic assays at a substrate concentration of 50 nM. IC₅₀ values were calculated using the Sigma plot program.

2.3. Hydrolysis of enkephalins

Essentially, enzymatic assays were carried out at 37°C in 100 mM of MES/NaOH pH 6.5 containing 1 µM captopril and 100 µM bestatin (inhibitors of angiotensin-converting enzyme and aminopeptidase, respectively) using 5 µg of substrate ([Leu⁵] enkephalin or [Leu⁵, Arg⁶] enkephalin) in a final volume of 50 µl. Reactions were stopped by freezing reaction tubes at -70°C. The reaction mixture was then fractionated at room temperature by reverse-phase HPLC on a µBondapak C18 column, 300 × 3.9 mm with a 2–40% gradient of acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. The absorbance of substrates and products was monitored at 214 nm. Peaks were identified by amino acid compositions. Percentage of hydrolysis was calculated by the estimation of peak area of non-hydrolysed substrates and YGG and YGGF products.

3. Results and discussion

NEP is known to hydrolyse substrates on the amino side of hydrophobic amino acid residues [25]. Although this S₁' specificity is relatively well defined, other subsite preferences are less obvious. For example, it has been suggested that NEP S₂' subsite prefers hydrophobic residues [11]. However, it can accommodate leucine, methionine, arginine or glycine lateral chains with minor differences in substrate specificity [8]. The best explanation for this apparent low S₂' specificity is that the substrate-enzyme interactions occurring with the peptide backbone on either side of P₂' residue could hide any subsite preference. This hypothesis is supported by our previous demonstration that Arg-102 forms a weak salt bridge or a hydrogen bond with the carbonyl/carboxyl group of the substrate P₂' residue, and that Asn-542 forms a hydrogen bond with the amide groups of the same residue (see Fig. 1) [13,14]. To assess the S₂' specificity we have created mutated NEPs in which Arg-102 and/or Asn-542 were replaced by Met and Gly, respectively. These mutations should prevent any enzyme interaction with the carbonyl/carboxyl and amide groups of the P₂' residue. The mutations were introduced in secNEP, a soluble form of NEP that has been shown previously to have

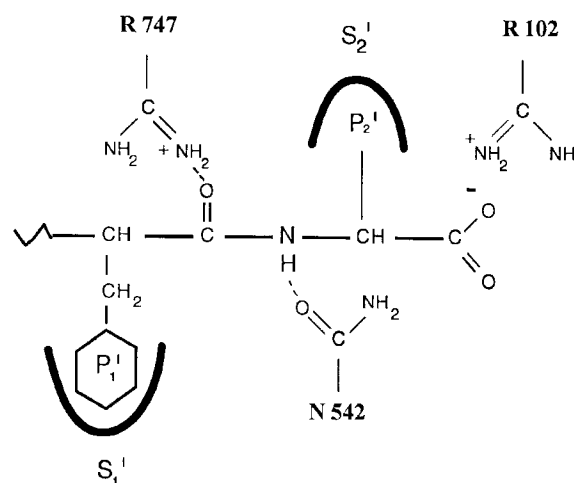


Fig. 1. Schematic representation of substrate interaction with S₁' and S₂' NEP subsites. NEP subsites are represented by grey half-circles. Substrate peptide backbone is in the centre and residues involved in the substrate binding are numbered. Notice that Arg-102 establishes a hydrogen bond or a weak salt bridge with the carbonyl/carboxyl side of the P₂' residue and Asn-542 establishes a hydrogen bond with the amino side of the P₂' residue.

enzymatic parameters similar to those of membrane-bound NEP [18]. The soluble form of the enzyme will be referred to as NEP throughout this paper.

3.1. Expression of native and mutated secreted NEPs in COS-1 cells

To obtain non-mutated and mutated enzymes, COS-1 cells were transfected with either vector pSVsec-NEP encoding the wild-type (non-mutated) enzyme [18] or vectors encoding R102M, N542G and R102M/N542G mutated enzymes. After recovery and concentration of the media, secreted NEPs were detected by immunoblotting and the band intensities compared to standard of known amounts by laser densitometer (result not shown). This analysis allowed on one hand to quantitate the amounts of each enzyme and on the other hand to confirm our previous observations that single and double mutations did not interfere with the biosynthesis, transport and secretion of the peptidases [13,14].

3.2. Kinetic parameters of non-mutated and mutated NEPs

Mutations R102M and N542G affected mainly the k_m values of the enzyme (Table 1) as was observed previously when these mutations were introduced in the membrane-bound form of NEP [13,14]. This observation confirms our previous conclusions that NEP active site located in the ectodomain of the enzyme functions independently from the rest of the protein [18]. The 2-fold increase in k_m value for R102M NEP and

Table 1
Kinetic parameters of non-mutated (wt) and mutated NEPs

	k_m (µM)	k_{cat} (min ⁻¹)	k_{cat}/k_m (min ⁻¹ /µM)	$\Delta\Delta G^a$ (kcal/mol)
WT	109 ± 7 ^b	1017 ± 49	9.33	—
R102M	240 ± 42	1257 ± 107	5.24	0.4
N542G	1399 ± 329	732 ± 81	0.523	1.8
R102M/N542G	2137 ± 146	412 ± 27	0.193	2.5

^a $\Delta\Delta G = -RT \ln [(k_{cat}/k_m) \text{ mutated NEP} / (k_{cat}/k_m) \text{ non-mutated NEP}]$.

^bValues are the mean of three different experiments (mean ± S.E.M.).

Table 2
Dipeptide inhibitions of non-mutated and mutated NEPs (IC_{50} μ M)

P ₁	P ₂	WT	R102M	N542G	R102M/ N542G
Phe	GLY	30.2 $\pm$ 8.5 ^a	1000 ^b	3000 ^b	NI ^c
Phe	ALA	6.6 $\pm$ 0.8	328 $\pm$ 24	541 $\pm$ 65	1285 $\pm$ 320
Phe	LEU	20.0 $\pm$ 8.9	107 $\pm$ 11	1000 ^b	1000 ^b
Phe	VAL	20.8 $\pm$ 1	34.9 $\pm$ 2.3	475 $\pm$ 52	597 $\pm$ 61
Phe	PHE	15.9 $\pm$ 2	83.2 $\pm$ 7.4	255 $\pm$ 33.4	310 $\pm$ 92
Phe	TRP	3.5 $\pm$ 1.2	5.5 $\pm$ 0.33	78.7 $\pm$ 9.5	194 $\pm$ 3.3
Phe	TYR	32.5 $\pm$ 3	157 $\pm$ 21	681 $\pm$ 96	1286 $\pm$ 445
Phe	ARG	2560 $\pm$ 130	582 $\pm$ 47	2500 ^b	659 $\pm$ 100
Phe	GLN	602.0	950.0	1500 ^b	1500 ^b

^aValues are the mean of three different experiments (mean $\pm$ S.E.M.)

^bDue to low solubility of dipeptides the IC_{50} could not be determined precisely

^cNI, Not inhibited.

the 13-fold increase for N542G NEP correspond to losses of binding energy of 0.4 and 1.8 kcal/mol, respectively (Table 1). These values are consistent with the loss of a weak salt bridge or one hydrogen bond in each mutant [26]. When both mutations were introduced at the same time in the enzyme, we observed an additive effect (21-fold) on the k_m value of NEP (Table 1). In contrast, the k_{cat} value decreased by only 2.5-fold (Table 1). These results confirm the involvement of Arg-102 and Asn-542 in substrate and inhibitor binding but indicate that Asn-542 contributes more to the interaction than Arg-102.

3.3. Inhibition of non-mutated and mutated NEPs with dipeptides

To determine NEP S_2' preference, we studied the inhibition of wild-type or mutated NEPs with dipeptides. These simple inhibitors contain a P₁' phenylalanine, essential for the interaction with the S_1' hydrophobic pocket of NEP and a variable P₂' residue (Phe-X). Inhibition of non-mutated NEP with these dipeptides suggests that the S_2' subsite prefers non-polar side chains (Table 2) and does not appear to easily accommodate charged (Arg) or polar (Gln) residues. The size of the side chain is not an important factor since similar IC_{50} values were obtained for Phe-Trp and Phe-Ala the two best inhibitors. Similarly, Phe-Gly and Phe-Tyr showed almost identical inhibition potencies.

When Arg-102 was replaced by Met, we observed a general increase in the IC_{50} values of the inhibitors, confirming the role of Arg-102 in substrate/inhibitor binding (Table 2). This

mutation affected Phe-Gly and Phe-Ala the most whereas dipeptides with larger side chains in P₂' were affected to a lesser extent. The IC_{50} of Phe-Gln did not increase dramatically. However, the value was close to 1 mM confirming again that the polar side chain of Gln is not well accepted. Interestingly, replacing Arg-102 by Met facilitates the interaction with Phe-Arg. Since replacing Arg by Met is an isosteric substitution, our observation suggests that the positive charge of Arg-102 interferes with the binding of Phe-Arg to NEP. As for the replacement of Arg-102 by Met, substitution of Gly for Asn-542 resulted in an increase in the IC_{50} values of the inhibitors. This increase was however larger than that observed with R102M NEP confirming that Asn-542 contributes more to the binding of these dipeptide inhibitors than Arg-102. With this mutant, Phe-Trp and Phe-Phe showed the best inhibition. Again Phe-Gly and Phe-Ala were the most affected by the mutation, and Arg is not well accepted in the P₂' of this mutated enzyme. When both Arg-102 and Asn-542 were mutated, the increase observed in the IC_{50} values was larger than for the single mutations reflecting the cooperation of Arg-102 and Asn-542 in NEP substrate/inhibitor binding. With this mutant Phe-Gly had no inhibitory effect, and Phe-Trp and Phe-Phe were again the inhibitors with the best IC_{50} values.

Inhibition of wild-type and mutated enzymes with dipeptide inhibitors has emphasized once again the importance of the interactions that Arg-102 and Asn-542 establish with the substrate or in this case with the inhibitor peptide backbone. Furthermore, our results show that as the interaction between the enzyme and the inhibitor peptide backbone is weakened

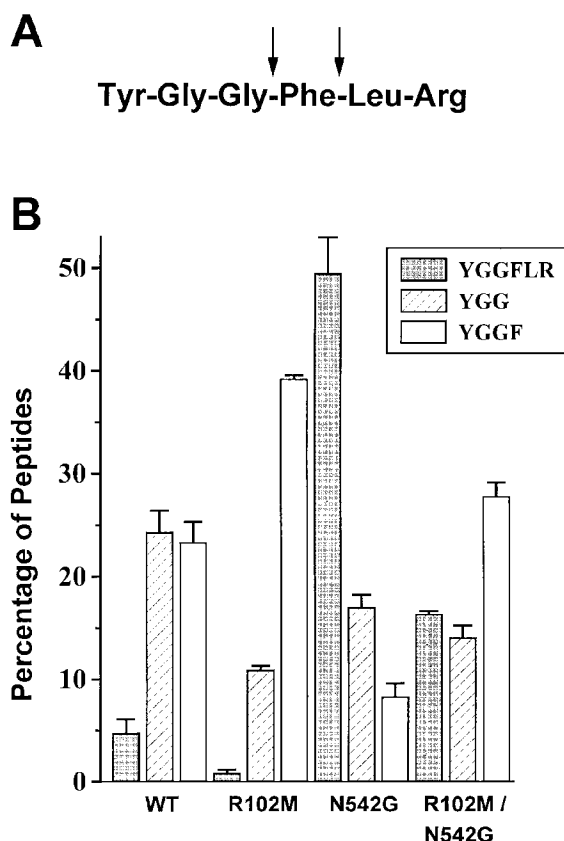


Fig. 2. Hydrolysis of [Leu⁵, Arg⁶] enkephalin by non-mutated and mutated NEPs. (A) Primary structure of [Leu⁵, Arg⁶] enkephalin and localisation of the cleavage sites (black arrows). (B) Percentage of intact substrate (dark boxes), YGG (stippled boxes) and YGGF (open boxes) peptides generated by hydrolysis of [Leu⁵, Arg⁶] enkephalin with non-mutated and mutated NEPs. Values are the mean of at least three different experiments.

by successive or concomitant mutations of Arg-102 and Asn-542, the nature of the P₂' side chains becomes more important for efficient binding. This is best demonstrated by the behaviour of Phe-Gly and Phe-Trp. While both inhibitors have similar potency with the non-mutated enzyme, R102M/N542G NEP is not inhibited by Phe-Gly at a concentration of 10 mM whereas Phe-Trp is still a good inhibitor.

3.4. Hydrolysis of [Leu⁵, Arg⁶] enkephalin

To confirm our findings, we studied the hydrolysis of [Leu⁵, Arg⁶] enkephalin. It is already known that NEP cleaves this substrate at the Gly³-Phe⁴ or Phe⁴-Leu⁵ bonds (Fig. 2A) with no apparent preference for a hydrophobic or a positively charged lateral chain in the P₂' position [8]. Fig. 2B shows that indeed NEP cleaves [Leu⁵, Arg⁶] enkephalin at both sites generating similar proportions of YGG and YGGF peptides (24.3 ± 2% and 23.3 ± 2%, respectively). NEP mutants also hydrolyse the elongated enkephalin at both sites. However, the ratio of YGG to YGGF varied for the different mutants (Fig. 2B). Both R102M NEP and R102M/N542G NEP preferred the Phe⁴-Leu⁵ bond over the Gly³-Phe⁴ bond. The former in a 4 to 1 ratio and the latter in a 2 to 1 ratio (Fig. 2B). In the case of N542G NEP, the Gly³-Phe⁴ bond was cleaved preferentially over the Phe⁴-Leu⁵ bond in a 2 to 1 ratio (Fig. 2B). In the same reaction conditions, non-mutated NEP, R102M NEP, N542G NEP and R102M/N542G NEP

cleaved approximately 80%, 60%, 85% and 70%, respectively, of [Leu⁵]enkephalin at the Gly³-Phe⁴ bond (results not shown).

These results, in agreement with the dipeptide inhibition studies, emphasize the role of Arg-102 of NEP in restricting access of the S₂' subsite to some residues such as arginine: in both mutants where Arg-102 was replaced by Met, Arg was preferred to Leu in the S₂' subsite. This preference for the Phe⁴-Leu⁵ bond may be explained by a better activity of NEP as a dipeptidyl carboxypeptidase than as an endopeptidase when both activities are possible [27,9]. In a previous study [13] we speculated that Arg-102 was a key feature in directing NEP dipeptidyl carboxypeptidase activity. The present study suggests that other structural features of NEP are also involved. Finally, our studies with dipeptide inhibitors as well as a previous report [11] have suggested that the S₂' subsite had a preference for a hydrophobic/aromatic side chain. This conclusion is not fully supported by the data obtained from the hydrolysis of [Leu⁵, Arg⁶]enkephalin. We believed the data should be interpreted as indicating that the S₂' subsite is a vast subsite that can accommodate large side chains. Stabilization of substrates or inhibitors in this S₂' subsite is accomplished by non-specific Van der Waals interactions between the side chain and the enzyme.

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