Active Site Mutations Change the Cleavage Specificity of Neprilysin

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Abstract

Neprilysin (NEP), a member of the M13 subgroup of the zinc-dependent endopeptidase family is a membrane bound peptidase capable of cleaving a variety of physiological peptides. We have generated a series of neprilysin variants containing mutations at either one of two active site residues, Phe⁵⁶³ and Ser⁵⁴⁶. Among the mutants studied in detail we observed changes in their activity towards leucine⁵-enkephalin, insulin B chain, and amyloid β₁₋₄₀. For example, NEP⁵⁶³I displayed an increase in preference towards cleaving leucine⁵-enkephalin relative to insulin B chain, while mutant NEP⁵⁴⁶E was less discriminating than neprilysin. Mutants NEP⁵⁶³L and NEP⁵⁴⁶E exhibit different cleavage site preferences than neprilysin with insulin B chain and amyloid β₁₋₄₀ as substrates. These data indicate that it is possible to alter the cleavage site specificity of neprilysin opening the way for the development of substrate specific or substrate exclusive forms of the enzyme with enhanced therapeutic potential.

Introduction

Neprilysin (NEP), also known as neutral endopeptidase 24.11, CD10, enkephalinase, and CALLA, is a member of the M13 subgroup of zinc-dependent endopeptidases [1]. NEP was originally discovered in rabbit kidney as a peptidase that cleaves insulin B chain [2]. Subsequent studies showed that NEP is widely expressed throughout mammalian tissues, including the lung, male genital tract, fibroblasts, various epithelia, and at neural synapses in the central nervous system [3–5]. The enzyme cleaves a variety of physiological substrates including bombesin-like peptides, amyloid β peptides (Aβ), leucine⁵ or methionine⁵-enkephalin, bradykinin, atrial natriuretic factor (ANF), and substance P [6–9]. NEP exhibits a preference for cleavage on the amino terminal side of hydrophobic residues [10].

Because of its multiple targets, NEP has been the focus of numerous studies attempting to modulate its activity for therapeutic purposes. One such target is the use of NEP to reduce Aβ peptide levels in Alzheimer’s disease, since the oligomerization of Aβ has been linked to the etiology of this disease [11]. Indeed, in studies with transgenic mice NEP expression decreases the level of Aβ [12–15] and ameliorates cognitive deficits typically attributed to AD [16]. In yet another application inhibitors of NEP were developed to block its “enkephalinase” activity to increase the concentration of enkephalins in the brain and thus their analgesic effect [17]. Peripherally expressed NEP may have a role in appetite control and obesity. NEP deficient mice become obese [18], while a peripherally administered NEP inhibitor that does not cross the blood-brain barrier increased food intake and subsequently led to obesity. Recently, an NEP inhibitor was shown to increase female genitalia blood flow in rabbits by preventing vasoactive intestinal peptide (VIP) cleavage [19]. This could potentially lead to the use of NEP as a therapeutic agent in the treatment of female sexual arousal disorder.

While methods to modulate NEP activity have displayed the potential for therapeutic use, they also reveal a paradox to their usage. For example, using NEP to lower Aβ may indeed decrease the amount of the target substrate; it may also have undesired consequences by removing other physiologically important products such as the enkephalins or vasopressin. Alternatively, inhibiting NEP to enhance opioid levels will likely cause an increase in Aβ, which would result in an increased risk in the development of Alzheimer’s disease.

A strategy to bypass the potential problems associated with the substrate promiscuity of NEP is to alter its specificity towards a target substrate thus reducing potential off-target effects. There is ample precedence to apply such a strategy. For example, substitutions within the active site of trypsin, although decreasing activity, shifted the relative preference for arginine versus lysine [20]. Similarly, a series of mutations in Rous sarcoma virus protease displayed altered amino acid preferences at particular substrate positions, allowing position-by-position control of substrate specificity [21]. Using thermolysin as a homology model, we were able to show that conversion of Val⁵⁷⁷ to Leu produced a form of NEP which reacted with substrates with small P1 residues essentially the same as wild-type enzyme, yet substrates containing bulky P1 residues exhibited a decreased Vmax with little change in Km [22]. This study, although limited in scope, demonstrated the...
feasibility of altering NEP substrate specificity. The nomenclature of Schecter and Berger (Schechter I, Berger A. (1968) Biochem. Biophys. Res. Commun. 32; 890-902) is used where residues of the substrate C-terminal to the site of cleavage are designated P1', P2', P3', etc. as they move away from the scissile bond and residues N-terminal to the scissile bond are designated P1, P2, P3, etc. as they move away from the scissile bond. The corresponding binding sites on the enzyme are designated S1', S2', S3', and S1, S2, S3, etc. respectively.

By analyzing the crystal structure of NEP in complex with the inhibitor phosphoramidon [23], we have initiated a rational design approach to mutate NEP active site targeting residues likely to interact with substrates. In this study, we explore NEP substrate specificity by generating NEP mutant libraries of two active site residues, Phe563 which is part of the S1' binding site and Ser546 which appears to contribute to the S2/S3 binding site. A number of these mutants displayed differential changes in activity toward physiological substrates including changes in cleavage site preferences. Together, these data support the hypothesis that amino acid changes in the active site of NEP can potentially give rise to therapeutically relevant forms of NEP.

Results and Discussion

Selection of sites for mutagenesis

Mutations were made at the Phe563 and Ser546 sites in a secreted form of human NEP (shNEP) expressed as a C-terminal hexahistidine fusion protein. The NEP crystal structure reveals that Phe563 forms part of the S1' substrate binding pocket believed to impart the preference for hydrophobic/aromatic P1' residues at this position, Figure S1. Phe563 is located in a coiled region just prior to the helix containing the active site residues. Ser546 is part of a β-sheet lining the substrate-binding site [23] and is positioned to interact with the P2 or P3 residues of a bound substrate on the carboxyl side of the scissile bond. Based on the NEP crystal structure, the position of both Phe563 and Ser546, and their conservation among species, we hypothesized that these residues contribute to substrate specificity.

Expression of mutant NEP

To test the contribution of Phe563 and Ser546 to catalysis we used degenerate oligonucleotides to construct NEP libraries in which we introduced amino acid substitutions at these positions. Substitutions made at Phe563 included valine, leucine, methionine, isoleucine, serine, histidine, aspartic acid, arginine, glutamine, asparagine, and lysine. Substitutions made at Ser546 included glutamate, lysine, threonine, glycine, arginine, and alanine. Individual mutant library members were transfected in HEK293 cells and analyzed for expression. Of the seventeen sequences examined, five mutants, NEP563L, NEP563V, NEP563M, NEP563I, and NEP563T, expressed at levels near to that of wild-type NEP and were selected for further purification and analysis. The low expression of other mutants appeared to be due to their cellular instability as they all produced similar amounts of mRNA, Figure S2, which did not correlate with protein expression nor did the poorly expressing mutants accumulate intracellularly. These results suggest that active-site residues Phe563 and Ser546 play a role in overall protein folding and/or stability and that the non-expressing mutants were likely degraded intracellularly.

The five expressing NEP mutants were purified by nickel affinity chromatography, and the amount of NEP present determined by Sypro ruby staining of SDS-PAGE gels. We initially compared Sypro Ruby and Western blot analysis for enzyme quantitation and obtained equivalent results with either method, Figure S3.

Reaction of NEP mutants with the synthetic substrate Glut-Ala-Ala-Phe-MNA

Activity assays were first performed using the synthetic peptide Glut-Ala-Ala-Phe-MNA. This substrate is cleaved between the Ala-Phe peptide bond and thus any effects of mutations on the cleavage at this site will be reflected in the reaction kinetics. We demonstrated that Glut-Ala-Ala-Phe-MNA hydrolysis by NEP and each of the studied mutants was completely inhibited by the relatively specific inhibitor, phosphoramidon at 100 μM, and the highly specific inhibitor CGS 24592 [24] at 10 nM, thus demonstrating that hydrolysis was attributed to NEP or its variant and not a contaminating protein.

Kinetic constants for mutants determined with Glut-Ala-Ala-Phe-MNA as substrate are presented in Table 1. These kinetic constants were derived under first-order assay conditions monitored in a continuous mode. The wild-type enzyme and the NEP563L mutant exhibited essentially the same specific activity of 46 and 44 pmoles/min/ng, respectively, while the NEP563I, NEP563V, NEP563M, and NEP563T mutant activities varied from ~25% to ~45% of the wild-type enzyme, Table 1. K_m values varied ~2.5 fold ranging from 51 to 118 μM, with V_max/K_m values varying three fold or less. Thus mutating Phe563 and Ser546 produce small but detectable affects on the cleavage of Glut-Ala-Ala-Phe-MNA confirming that these residues contribute to catalysis.

Table 1. The specific activity towards glutaryl-Ala-Ala-Phe-MNA cleavage is reduced in NEP mutants.

| Specific Activity | K_m | K_inhibitor B chain |
|------------------|-----|---------------------|
| (pmoles/min/ng)  | (μM) | (μM) |
| NEP              | 45.9±2.5 | 51±11 | 1.9±0.2 |
| NEP563L          | 17.7±0.6 | 83±5 | 2.5±0.9 |
| NEP563V          | 43.6±0.1 | 81±11 | 1.5±0.3 |
| NEP563M          | 19.2±0.4 | 87±8 | 1.2±0.1 |
| NEP563I          | 56.2±0.3 | 51±10 | 1.0±0.1 |
| NEP563T          | 20.1±0.6 | 74±10 | ND |
| NEP563A          | ND* | 118±7 | ND |
| NEP563T*         | ND* | 73±6 | ND |

All assays were conducted at 37°C at in 20 mM MES buffer, pH 6.5. ND = not determined.
*The concentrations of NEP563A and NEP563T were too low to quantify, however, they were active enough to determine K_m.

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The specific activities of the various mutants with the physiological substrate leu-ENK showed a similar pattern as observed with Glut-Ala-Ala-Phe-MNA. NEPF563L had near wild-type levels of activity for both Glut-Ala-Ala-Phe-MNA and leu-ENK, while NEPF563V, NEPF563I, and NEPS546E all showed approximately 40–60% activity towards these substrates, and NEPF563V exhibited a 75–85% decrease in activity towards both. Thus the effects of these mutations can be attributed to those produced for cleaving N-terminal to single phenylalanine residue.

Insulin B chain and Aß1–40 contain multiple cleavage sites. Cleavage at any one of these sites will result in peptide disappearance as determined by HPLC. Compared to wild-type NEP, mutants NEPF563V, NEPS546E, NEPF563I, and NEPF563L all exhibited reduced hydrolysis rates for insulin B chain (p values of 0.03 or lower), whereas with NEPF563I, the hydrolysis rate was higher (p = 0.03). With Aß1–40 as substrate all of the mutants showed reduced rates of hydrolysis (all exhibited p values<0.05).

When the relative cleavage rates for insulin B chain, Aß1–40, and leu-ENK were compared between NEP and the various mutants by using insulin B chain as an alternate substrate (competitive) inhibitor of Glut-Ala-Ala-Phe-MNA hydrolysis. We found that there was no dramatic change in the Kᵦ for any of the mutants, with variations of two fold or less (Table 1).

Degradation of insulin B chain by wild-type NEP was analyzed by reverse-phase HPLC following the appearance of each product as a function of time, Figure 1. Product peaks were collected and subsequently analyzed by mass spectrometry to determine their identities. As shown in Table 4 and the insert in Figure 1 this analysis identified seven cleavage sites. Based on the order of appearance of each peak, it is likely that insulin B chain1–10, insulin B chain11–11, insulin B chain11–14, and its partner peak insulin B chain15–30, insulin B chain17–30, and insulin B chain24–30 are all products of primary cleavages. These products appear at the first time point when approximately 15% hydrolysis of insulin B chain had occurred (30 minutes). At 30% hydrolysis (90 min.), peaks corresponding to insulin B chain1–5 and insulin B chain7–24 are observed. Peaks for insulin B chain1–16 and insulin B chain13–23 become apparent at >30% insulin B chain hydrolysis. It should be noted that the expected product peaks insulin B chain11–30 and insulin B chain12–30, the partner products of insulin B chain10 and insulin B chain11, respectively, were identified by mass spectrometry; however the peaks never accumulated significantly above the baseline throughout the incubation. It is likely that these are transient products that are subsequently cleaved contributing to other product peaks (i.e. insulin B chain24–30).

### Table 2. Rates of hydrolysis of physiological peptides by NEP mutants.

|            | leu-ENK     | Insulin B Chain | Aß1–40     |
|------------|-------------|-----------------|------------|
|            | pmole/min/ng| pmole/min/ng    | fmole/min/ng|
| NEP        | 1.90±0.23   | 0.86±0.13       | 198±12     |
| NEPF563L   | 1.57±0.32   | 1.17±0.18       | 116±4      |
| NEPF563M   | 1.21±0.22   | 0.21±0.01       | 85±3       |
| NEPF564E   | 1.12±0.12   | 0.58±0.07       | 93±8       |
| NEPF563I   | 1.06±0.17   | 0.13±0.02       | 43±1       |
| NEPF563V   | 0.31±0.02   | 0.06±0.02       | 14±1       |

Hydrolysis was carried out at 37°C at in 20 mM MES buffer, pH 6.5. Substrate concentrations were 15 μM insulin B chain, 24 μM Aß1–40, and 64 μM leu-ENK. Activity was determined by following the disappearance of substrate by HPLC. Each reaction was run in at least triplicate. Statistical analysis was conducted using a two-tailed paired t-test with Prism4 software.

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### Table 3. Leu-Enkephalin and Insulin B chain dual substrate assays.

| Mutant NEP | leu-ENK | Insulin B Chain | Ratio leu-ENK |
|------------|---------|-----------------|---------------|
|            | pmole/min/ng | pmole/min/ng | insulin B Chain |
| NEP        | 1.16±0.06 | 0.20±0.04       | 0.23          |
| NEPF563V   | 0.23±0.03 | 0.04±0.01       | 0.67          |
| NEPF563I   | 0.97±0.06 | 0.19±0.03       | 0.20          |
| NEPF563M   | 0.69±0.11 | 0.11±0.02       | 0.52          |
| NEPS546E   | 0.62±0.03 | 0.02±0.01       | 0.15          |
| NEPF564E   | 0.76±0.14 | 0.25±0.01       | 0.43          |

The numbers in parenthesis indicate activity relative to the uninhibited values. Reactions were carried out at 37°C at in 20 mM MES buffer, pH 6.5 containing 15 μM insulin B chain and 64 μM leu-ENK. Activity was measured and statistical analysis carried out as in Table 2.

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The specificity of the various mutants with the physiological substrate leu-ENK showed a similar pattern as observed with Glut-Ala-Ala-Phe-MNA. NEPF563L had near wild-type levels of activity for both Glut-Ala-Ala-Phe-MNA and leu-ENK, while NEPF563V, NEPF563I, and NEPS546E all showed approximately 40–60% activity towards these substrates, and NEPF563V exhibited a 75–85% decrease in activity towards both. Thus the effects of these mutations can be attributed to those produced for cleaving N-terminal to single phenylalanine residue.

Insulin B chain and Aß1–40 contain multiple cleavage sites. Cleavage at any one of these sites will result in peptide disappearance as determined by HPLC. Compared to wild-type NEP, mutants NEPF563V, NEPS546E, NEPF563I, and NEPF563L all exhibited reduced hydrolysis rates for insulin B chain (p values of 0.03 or lower), whereas with NEPF563I, the hydrolysis rate was higher (p = 0.03). With Aß1–40 as substrate all of the mutants showed reduced rates of hydrolysis (all exhibited p values<0.05).

Identification of NEP cleavage sites in insulin B chain

We next looked in more detail on the effect of the NEPF563 and NEPS546 mutations on the hydrolysis rates at individual cleavage sites in insulin B chain. We first tested for a change in the affinity of insulin B chain for mutant NEPs by using insulin B chain as an alternate substrate (competitive) inhibitor of Glut-Ala-Ala-Phe-MNA hydrolysis. We found that there was no dramatic change in the Kᵦ for any of the mutants, with variations of two fold or less (Table 1).

Degradation of insulin B chain by wild-type NEP was analyzed by reverse-phase HPLC following the appearance of each product as a function of time, Figure 1. Product peaks were collected and subsequently analyzed by mass spectrometry to determine their identities. As shown in Table 4 and the insert in Figure 1 this analysis identified seven cleavage sites. Based on the order of appearance of each peak, it is likely that insulin B chain1–10, insulin B chain11–11, insulin B chain11–14, and its partner peak insulin B chain15–30, insulin B chain17–30, and insulin B chain24–30 are all products of primary cleavages. These products appear at the first time point when approximately 15% hydrolysis of insulin B chain had occurred (30 minutes). At 30% hydrolysis (90 min.), peaks corresponding to insulin B chain1–5 and insulin B chain7–24 are observed. Peaks for insulin B chain1–16 and insulin B chain13–23 become apparent at >30% insulin B chain hydrolysis. It should be noted that the expected product peaks insulin B chain11–30 and insulin B chain12–30, the partner products of insulin B chain10 and insulin B chain11, respectively, were identified by mass spectrometry; however the peaks never accumulated significantly above the baseline throughout the incubation. It is likely that these are transient products that are subsequently cleaved contributing to other product peaks (i.e. insulin B chain24–30).

Analysis of NEP mutant cleavage of insulin B chain

As a representative of the NEPF563 and NEPS546E mutants, we compared the cleavage profile of NEPF563L and NEPS546E to that of NEP using time course experiments, Figure 2. By adjusting the amount of NEP mutant used, the rate of hydrolysis of insulin B chain by all NEP forms was virtually identical. The overall...
cleavage profile at 30% substrate hydrolysis revealed that all of the product peaks observed with NEP are present with the mutant enzymes indicating that there were no unique or missing cleavage sites between NEPF563L, NEPS546E and NEP, Figure 2A. Since rates were based on peak areas measured at 214 nm, which in turn is dependent on both the number of peptide bonds and the number of aromatic residues within a given peptide, only the observed rates of change for a particular peptide product can be compared between enzyme forms. A comparison of the rate of change of different peaks within the same enzyme form or between enzyme forms is not valid under our conditions of analysis.

NEP mutations affect cleavage sites preferences in insulin B chain

Table 5 and Figure 2B,C show the rates of product accumulation normalized to the amount of NEP protein present. Relative to wild-type NEP the overall rate of hydrolysis of insulin B chain is slightly increased in NEPF563L and slightly decreased in NEPS546E, Table 2. Thus one scenario is that all sites in insulin B chain would be cleaved at the same relative rate compared to wild-type NEP. Alternatively, the introduced mutations may differentially affect specific cleavage sites. The data in Table 5 clearly shows the latter scenario with differential effects of mutations on specific cleavages. NEPS546E cleaves insulin B chain at an overall rate 0.7 times that of NEP, however it is clear that cleavage at A14-L15 is nearly identical between NEP and this mutant. Cleavage at H5-L6 is well below the overall insulin B chain rate of 0.7 (Figure 2B), while cleavages at H10-L11, Y16-L17, and G23-F24 are all slightly slower than the expected 0.7 times the wild-type rate. NEPF563L cleaves insulin B chain at a rate 1.4 times that of NEP. Similar to that seen with NEPS546E, NEPF563L products produced from single cleavage sites exhibit noticeably different rates compared to NEP, Figure 2C. Cleavage at A14-L15, H5-L6, and G23-F24 are close to the expected 1.4 times faster that of NEP, but cleavage at H10-L11, L11-V12, and Y16-L17 are slower than NEP, (0.8 times, 0.2 times, and 0.7 times the NEP rate respectively, rather than the overall 1.4 times faster than the NEP rate).

Based on the data in Table 5 the elevated activity of NEPF563L towards insulin B chain can likely be attributed to an increased rate of cleavage at the primary cleavage site A14-L15. Although this cleavage involves a leucine residue, the finding that cleavage at Y16-L17 is slower than with wild-type NEP shows the enhanced cleavage at A14-L15 is not due to simply the F563L mutation.

Table 4. Products of insulin B chain hydrolysis by NEP.

| Retention Time (min.) | Insulin B Chain Fragment | Expected Mass | Observed Mass | Cleavage Site |
|-----------------------|--------------------------|---------------|---------------|---------------|
| 9.1                   | 1–5                      | 643.31        | 643.24        | H5-L6         |
| 18.3                  | 1–10                     | 1188.48       | 1188.39       | H10-L11       |
| 22.7                  | 17–24                    | 927.42        | 927.35        | Y16-L17       |
| 24.3                  | 1–11                     | 1301.6        | 1301.47       | L11-V12       |
| 24.7                  | 15–23                    | 1056.51       | 1056.42       | A14-L15       |
| 25.4                  | 1–14                     | 1600.75       | 1600.54       | A14-L15       |
| 26.2                  | 24–30                    | 872.44        | 872.39        | G23-F24       |
| 29.1                  | 17–30                    | 1634.79       | 1634.64       | Y16-L17       |
| 31.9                  | 1–16                     | 1876.89       | 1876.75       | Y16-L17       |
| 33.3                  | 15–30                    | 1910.93       | 1910.84       | A14-L15       |
| 33.6                  | 12–30                    | 2210.87       | 2209.93       | L11-V12       |
| 35.5                  | 11–30                    | 2323.17       | 2323.06       | H10-L11       |
| 36.6                  | 1–30                     | 3493.67       | 3493.52       | (insulin B chain) |

NEP mediated hydrolysis was carried out as described in Table 2. The reaction was stopped by adding 10 μL of 5% TFA when approximately half of the substrate had been hydrolyzed (180 min). The acidified reaction mixture was subjected to HPLC as described in figure 1, individual peaks were collected and identified by mass spectral analysis.

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Figure 1. Time course for NEP mediated hydrolysis of insulin B chain. Time course assays were conducted by incubation of NEP with 15 μM insulin B chain in 20 mM MES buffer, pH 6.5, at 37 C. A 100 μL aliquot was removed at each time point and 10 μL of 5% trifluoroacetic acid (TFA) was added to stop further hydrolysis. 95 μL were injected into a Vydac C4 column and developed as described in Materials and Methods. Each product was isolated and subjected to mass spectral analysis. Numbers under each peak indicate the identification of the peptide by sequence. Peaks without numbers were not identified.
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producing enhanced reactivity toward leucine, but more likely an effect of neighboring residues.

Identification of NEP cleavage sites in Aβ1–40

To further study the effect of mutations on cleavage site specificity, time course assays were also performed for the hydrolysis of the physiological substrate Aβ1–40. Like the analysis of insulin B chain, a time course assay was first done with NEP to identify cleavage products, Figure 3 and Table 6. After 15% hydrolysis of Aβ1–40 (150 min.) by NEP there were six discernable product peaks corresponding to Aβ1–16, Aβ1–17, Aβ10–17, Aβ20–28, Aβ20–29, and Aβ20–30. At 25% hydrolysis (240 min.), peaks corresponding to Aβ1–9, Aβ4–16, and Aβ4–17 were observed, while at 40% hydrolysis peaks Aβ4–9 and Aβ10–16 appeared. Peak Aβ12–17 was the last peak to be observed at 45% hydrolysis of Aβ1–40 (360 min.). Missing from the HPLC analysis were the C-terminal products resulting from the cleavages at K28-G29, G29-A30, and A30-I31. These products are derived from the trans-membrane region of the amyloid precursor protein (APP) from which Aβ is formed and are rather hydrophobic. It is likely that these peptides were not eluted in the gradient we employed.

Two other studies have identified NEP cleavage sites within Aβ1–40 [25,26]. These studies as well as the current study all detected cleavages at Gβ9-Y10, Fβ19-F20, and Aβ30-I31. Cleavages at Kβ28-Gβ29 and Gβ29-Aβ30 were detected in this study as well as by Leissring et al. [25,26]. In the current study additional unreported cleavage sites at Eβ11-Vβ12, Kβ16-Lβ17, and Lβ17-Vβ18 were detected. These were previously pointed out as potential cleavage sites, but were not observed [25,26]. Both Leissring and Howell identified cleavage at Gβ33-Lβ34, yet this cleavage was not found in this study. These differences likely reflect differences in the resolution of the Aβ1–40 cleavage products on the HPLC columns used and gradient conditions.

Analysis of specific cleavage sites in Aβ1–40

We next compared the Aβ1–40 cleavage profile of the NEPF563L and NEPS546E mutants to that of wild-type NEP, Figure 4. Since NEP, NEPF563L and NEPS546E cleave Aβ1–40 at different rates, the amount of the mutant enzymes used in the reaction was as before adjusted in order to analyze products formed at the same fraction of degradation. Interestingly, although all wild-type peaks were present in the NEPS563E mutant, the peak corresponding to Aβ12–17 was not present among the hydrolysis products of the NEPF563L mutant.

Similar to the analysis conducted with insulin B chain, the linear rate of product accumulation was determined for each peak and normalized to that of NEP for mutant NEPS546E, Figure 2. Changing the Cleavage Specificity of Neprilysin

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Figure 2. Comparison of insulin B chain cleavage between NEP, NEPF563L, and NEPS546E. A. HPLC profile of insulin B chain cleaved by NEP and NEP mutants at 30% hydrolysis. B. Rates of peak accumulation at each cleavage site normalized to that of NEP for mutant NEPS546E. C. Rates of peak accumulation at each cleavage site normalized to that of NEP for mutant NEPF563L. Dotted lines indicate the overall rate of hydrolysis of insulin B chain from Table 2. Reactions were carried out at 37°C with 15 μM insulin B chain in 20 mM MES, pH 6.5.
multiple cleavage events. Out of the 12 identified product peaks, only 3 could be produced by a single cleavage. Rates for all cleavages were calculated and are given in Figure 4, B–D, but only the three putative primary cleavage sites can be compared.

NEPS546E hydrolyzes Aβ1–40 at an overall rate 0.5 times that of wild-type NEP. Two of the three products resulting from a possible single cleavage by NEPS546E exhibit a higher than predicted rate of cleavage. Cleavage at K16-L17 producing Aβ1–16 is identical rather than half the wild-type rate while cleavage at L17-V18 producing Aβ1–17 was approximately 70% rather than 50% of the NEP rate. The remaining single cleavage site at G9-Y10 shows a slower rate being about 30% that of wild-type enzyme. Thus it would appear that all three of these cleavages contribute to the overall rate and together produce an average rate 0.5 times that of NEP.

Cleavage at L17-V18 is approximately three times faster for NEPS546E compared to NEP563L. Thus cleavage at L17-V18 for mutant NEPS546E is 70% of the wild-type NEP whereas cleavage at this bond for the NEP563L is 25% of NEP. In order to account for this finding there must either be an undetected cleavage that is significantly reduced in NEPS546E or more likely that NEPF563L exhibits a unique cleavage pattern that yields these products.

The rate of appearance of Aβ1–16 is linear over the entire 360-minute time course for all three enzymes. Aβ1–17, on the other hand, shows a linear increase with both NEPF563L and NEPS546E, but is non-linear with wild-type NEP showing very little increase after 150 min. This is consistent with Aβ1–17 being further metabolized by NEP, most likely by being cleaved at G9-Y10 giving rise to Aβ1–9 and Aβ10–17, both of which show higher peak areas in NEP than with either mutant. In contrast the absence of an obvious reduction of Aβ1–17 in the reaction of NEPF563L and NEPS546E suggests these mutants cleave the G9-Y10 bond at a much slower rate.

Although NEPF563L did not produce a discrete Aβ1–9 peak, it appears to cleave the G9-Y10 bond as evidenced by the presence of the products Aβ1–9 and Aβ10–17. Since Aβ1–9 is absent in the NEPF563L profile but Aβ10–17 is present, the cleavage by NEPF563L at G9-Y10 is likely dependent on the cleavage at A3-F4. Aβ1–9 is not further degraded by hydrolysis at the A3-F4 site with both NEP and NEPS546E as evidenced by its linear increase as a function of time. If hydrolysis occurred at the A3-F4 bond of the Aβ1–9 product, one would expect either no time dependent increase or a decrease in the Aβ1–9 peak.

The finding that most of the observed products of Aβ1–40 cleavage result from multiple cleavages, even for early time points, would suggest that NEP is processive in its cleavage of Aβ1–40 and can make several cleavages before product release. Whether the

| Table 5. Relative rates of accumulation of peaks generated by the NEP and NEP mutant dependent hydrolysis of insulin B chain. |
|---------------------------------------------------------------|
| **Single Cleavage**                                           |
| **Peak** | NEP | S546E | F563L |
| **Cleavage Site** | **D area/min/ng** | **D area/min/ng** | **D area/min/ng** |
| H5-L6    | 1–5  | 59    | 12    | 83     |
| H10-L11  | 1–10 | 163   | 87    | 135    |
| L11-V12  | 1–11 | 88    | 30    | 16     |
| A14-L15  | 1–14 | 167   | 145   | 266    |
|          | 15–30| 135   | 116   | 282    |
| Y16-L17  | 1–16 | 31    | 17    | 25     |
|          | 17–30| 171   | 81    | 112    |
| G21-L24  | 24–30| 268   | 142   | 290    |
| **Two Cleavages**                                           |
| **Peak** | NEP | S546E | F563L |
| **Cleavage Sites** | **D area/min/ng** | **D area/min/ng** | **D area/min/ng** |
| A14-L15, G21-L24 | 15–23 | 23    | 26    | 64     |
| Y16-L17, F24-L25 | 17–24 | 33    | 18    | 25     |

Time course assays were carried out by incubation of NEP with insulin B chain using conditions as described in Table 2. At 0, 30, 60, 90, 120, and 180 min, aliquots of 100 μL were removed followed by the addition of 10 μL of 5% TFA to stop further hydrolysis. Each reaction mixture was subjected to HPLC analysis as in Table 4 and peak areas measured. The rate of accumulation for each peak was calculated from the linear phase of the reaction.

Figure 3. Time course of NEP mediated hydrolysis of Aβ1–40. Time course measurements were carried out by incubation of NEP with 24 μM Aβ1–40 in 20 mM MES, pH 6.5, at 37°C. 100 μL aliquots were removed at each time point and 10 μL of 5% TFA was added to stop the reaction. Samples were analyzed as described in Figure 1. Numbers under each peak indicate the identification of the peptide by sequence.

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Table 6. Identification of products resulting from the hydrolysis of Aβ1–40 by NEP.

| Retention Time (min.) | Amyloid ß peptide fragment | Expected Mass | Observed Mass | Cleavage Site |
|-----------------------|-----------------------------|---------------|---------------|---------------|
| 7.4                   | 4–9                         | 717.32        | 717.30        | E^2-g^4-G^5-Y^10 |
| 10.3                  | 10–16                       | 939.46        | 939.44        | G^9-Y^16-K^16-L^17 |
| 14.7                  | 1–9                         | 1032.43       | 1032.43       | G^2-Y^10 |
| 15.8                  | 12–17                       | 760.43        | 760.44        | E^11-Y^12-L^17-V^18 |
| 17.2                  | 20–28                       | 964.45        | 965.46        | F^15-F^20-V^28-G^29 |
| 17.4                  | 20–29                       | 1022.44       | 1022.47       | F^19-F^20-V^29-A^30 |
| 18.2                  | 20–30                       | 1093.50       | 1093.52       | F^15-F^20-V^28-G^29 |
| 19.0                  | 4–16                        | 1638.76       | 1638.76       | E^3-F^4-K^16-L^17 |
| 19.8                  | 1–16                        | 1953.87       | 1953.83       | K^16-L^17 |
| 20.4                  | 10–17                       | 1052.54       | 1052.54       | G^2-Y^10-L^17-V^18 |
| 22.3                  | 4–17                        | 1751.85       | 1751.81       | E^3-F^4-L^17-Y^18 |
| 23.1                  | 1–17                        | 2066.96       | 2066.92       | L^17-V^18 |
| 37.4                  | 1–40                        | 4327.00       | 4327.15       | (Aβ1–40) |

NEP mediated hydrolysis was carried out as described in Table 2. The reaction was stopped by adding 10 μL of 5% TFA when approximately half of the substrate had been hydrolyzed (360 min.). The acidified reaction mixture was subjected to HPLC analysis as described in Table 4. Each peak was isolated and subjected to mass spectral analysis.

Changing the Cleavage Specificity of Neprilysin

Enzyme does this with both C-terminal and N-terminal products is not clear and how the products are reoriented in the active site for additional cleavages is also unclear. However, the alternative explanation for observing products derived from multiple cleavages requires an extremely high affinity of the product peptide to be bound and cleaved in the presence of a large amount of unreached Aβ1–40. The processive model is consistent with the overall structure of the enzyme, which has only a small opening leading to the large, enclosed chamber that borders the active site. Once a peptide diffuses through the narrow opening, it is likely that it and product peptides are retained in the enclosed chamber sufficiently long enough for multiple active site binding events to occur.

Of the eleven substitutions made at Phe563 only four produced enzyme of sufficient stability to be studied. The four residues that did produce stable forms of NEP all represented conservative change to hydrophobic residues, whereas the other non-conservative or semi-conservative changes produced unstable enzyme forms. This suggests position 563 likely serves as an important anchor residue in the folding of the enzyme, and interaction of a hydrophobic residue at this position with other hydrophobic and aromatic residues is required. Changes at Ser546 produced more variable results in terms of enzyme expression, and this position is therefore likely less critical in folding.

Amino acid substitutions at both Phe563 and Ser546 affected the cleavage pattern of NEP. Phe563 forms part of the S1’ substrate binding pocket and helps define the specificity of NEP for hydrophobic/aromatic P1’ residues, thus changing this amino acid would likely affect cleavage specificity. Ser546 is positioned to contribute to the S2/S3 binding site, although this registration is more speculative, since subsites N terminal to the scissile bond are not defined by available structures with bound inhibitors. Although selectivity is less stringent at these positions, we have previously obtained evidence that residues N terminal to the scissile bond, particularly the S1 subsite, also contribute to substrate specificity [27] and this study certainly supports a role for the P2/P3 peptide positions in selectivity.

Of the two mutants studied in detail no changes in the peptide bonds that were cleaved were observed, but the relative rates of cleavage were affected by substitution at both Phe563 and Ser546. In general substitution of leucine for phenylalanine at the S1’ site either had no effect or increased the rate at which cleavage occurred with a P1’ leucine or phenylalanine residue, but significantly decreased the rate when valine occupied the P1’ position. Since the NEP563L mutation substitutes a smaller residue in the hydrophobic S1’ subsite, this result can be rationalized on the basis that the relatively small valine at P1’ leaves an unfavorable gap upon substrate binding. Substitution of glutamate for serine at the S2/S3 position is an important role in selectivity. It is possible given the complex nature of the observed position that positioning of the N terminal side of the substrate peptide may vary in a sequence dependent manner.

Taken together this study shows that a single amino acid substitution within the active site of NEP can cause changes in cleavage site preference, which strongly supports the notion that it may be possible to alter the NEP active site to generate substrate specific variants that will be useful therapeutically.

Materials and Methods

Mutagenesis and production of expression vectors

NEP variants were constructed as gene segment cassette modules using degenerative oligonucleotide primers to introduce sequence diversity by PCR. Individual mutation cassettes were inserted into the pCDNA-shNEP-CHis (SacII+PstI) expression vector, a re-engineered pCDNA-3.1 vector with a silent SacII mutation introduced 5’ to the active site region within a secreted form of the human NEP (shNEP) coding sequence and a C-terminal hexahistidine affinity tag. Two silent mutations were made, using the Quickchange® II site directed mutagenesis kit (Stratagene) to eliminate additional PstI sites and facilitate cassette subcloning of the shNEP gene. A 3 kb fragment of lambda “stuffer” DNA was inserted between the SacII and PstI sites to allow gene segment cassette subcloning while eliminating wild-type sequences from being selected [27,28]. Non-polar substitutions at Phe563 were initially cloned into and sequenced using the pBPG1 vector for expression in yeast [29]. However, low enzyme yields from yeast led to the removal of the identified clones from the pBPG1 vector and subcloning into the pDNA-shNEP-CHis- (SacII-3kbstuffer-PstI) vector. PCR based mutagenesis was used to create NEP variants using degenerative primers containing appropriate restriction sites. For making polar substitutions at Phe563 the 5’ primer contained a PstI restriction site and the 3’ primer contained an NcoI restriction site to facilitate movement from the pBPG1 vector to the pDNA-shNEP-CHis- (SacII-3kbstuffer-PstI) vector. The degenerative primers (IDTDNA, Corvalville, IA) used were:

- 5’-gatgcgtgtcagctagcatgtg-3’ (S546 reverse primer)
- 5’-gggaggctcgggatccgtcgggagcagatagttttcgaATAAG-3’ (S546 forward primer)
- 5’-cactttgctgccccVISTTatgtcggaggatcagtcac-3’ (F563 forward primer for non polar residues)
- 5’-cactttgctgccccVISTTatgtcggaggatcagtcac-3’ (F563 forward primer for polar residues)
- 5’-ccacggcagtcggtgaattc-3’ (F563 reverse primer)
Expression and purification

NEP protein was expressed in HEK293T cells transfected with the pcDNA vector described above. Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) containing 10% FBS and 44 mM NaHCO₃ added as a supplement. For transfections, Polyfector (BamaGen Bioscience) and plasmid DNA were incubated at room temperature for 20 min in serum free DMEM media and then added to HEK293T cells in the DMEM media. The media was replaced with serum free DMEM 12–14 hours post transfection, and collected 72–96 hours post transfection. To the media was added 1 M Tris-HCl, pH 7.4 to a final concentration of 50 mM and the secreted enzyme was then purified on a His-Select Affinity Agarose Column (Sigma). The affinity purification step yielded enzyme with the purity dependent on the level of NEP expression. Activities measured in this study were attributed to NEP, since a mock transfection and purification resulted in no activity toward any of the substrates tested and NEP inhibitors eliminated all activity. We estimated the amount of NEP protein present by running the purified preparations on 8% or 10% SDS-PAGE gels along with purified NEP as a standard. The gels were stained with Sypro Ruby dye scanned on a Typhoon 9400 Imager, and quantified with Image Quant 5.2 software. In preliminary experiments the gel was transfered to a polyvinylidene fluoride (PVDF) and subject to Western blot analysis using goat anti-mNEP at 1:1000 (R&D systems) as the primary antibody and anti-goat IRDye800 at 1:20,000 (Rockland) as the secondary antibody. Probed membranes were imaged using an Odyssey infrared imager and Odyssey 2.1 software. Intensities of each band were analyzed with Image Quant 5.2 software. Data was analyzed using Prism4 software.

Activity assays

NEP activity was routinely assayed using the fluorogenic peptide glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide (Glut-Ala-Ala-Phe-MNA, Sigma) [30]. Reactions of 400 μl contained 100 mM Glut-Ala-Ala-Phe-MNA, 1 μg of aminopeptidase [31] and 15 to 100 ng of NEP or mutant NEP depending on their activity in 20 mM MES buffer, pH 6.5. Activity was monitored with a Spectra Max Gemini XS plate reader using an excitation wavelength of 340 nm and an emission wavelength of 425 nm. Reaction specificity was determined using the NEP inhibitors.

Figure 4. Comparison of Aβ₁₋₄₀ cleavage between NEP, NEP^{F563L}, and NEP^{S546E}. A. HPLC cleavage profile of Aβ₁₋₄₀ cleavage by NEP and NEP mutants at ~30% hydrolysis. B. Rates of peak accumulation at each cleavage site normalized to that of NEP for mutant NEP^{S546E}. C. Rates of peak accumulation at each cleavage site normalized to that of NEP for mutant NEP^{F563L}. Dotted lines indicate the overall rate of hydrolysis of Aβ₁₋₄₀ from Table 2. Reactions were carried out at 37°C with 15 μM Aβ₁₋₄₀ in 20 mM MES, pH 6.5.
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Table 7. Accumulation rates of products of NEP dependent cleavage of Aβ1–40.

| Cleavage Site | Peak | NEP | SS546E | F563L |
|--------------|------|-----|--------|-------|
| β2–Y10       | 1–9  | 7   | 2      | 0     |
| K16-L17      | 1–16 | 22  | 22     | 17    |
| L17-V18      | 1–17 | 75  | 51     | 22    |

Dual Cleavages

| Peak | NEP | SS546E | F563L |
|------|-----|--------|-------|
| A3-F4  | 4–9 | 10    | 5      |
| A3-F4  | 4–16 | 7    | 5      |
| A3-F4  | 4–17 | 9    | 12     |
| G5-V10 | 10–16 | 4   | 5      |
| G5-V10 | 10–17 | 18  | 8      |
| E11-V12 | 12–17 | 3   | 2      |
| F19-F20 | 20–28 | 11  | 5      |
| F19-F20 | 20–29 | 16  | 6      |
| F19-F20 | 20–30 | 34  | 15     |

Time course assays were carried out by incubation of NEP with 24 μM Aβ1–40 using reaction conditions as described in Table 5. At 0, 60, 150, 240, and 360 min, aliquots of 100 μL were removed followed by the addition of 10 μL of 5% TFA to stop further hydrolysis. Each reaction mixture was subjected to HPLC analysis as in Table 5 and peak areas measured. The rate of accumulation for each peak was calculated from the linear phase of the reaction.

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phosphoramidon and CGS 24592 [24], the latter being a highly specific and potent inhibitor.

Kinetic Analysis

Kinetic constants for NEP and its mutants were obtained using the assay conditions noted above, but with Glut-Ala-Ala-Phc-MNA varied from 20 to 500 μM. Typically 12 data points were obtained. The data were fit to the Michaelis-Menten equation using Prism4 software. The Kᵢ for insulin B chain was obtained from the equation: ID50 = Kᵢ (1/rate versus [insulin B chain]) using Prism4 software. The Kᵢ for insulin B chain was obtained by measuring the rate of Glut-Ala-Ala-Phe-MNA hydrolysis in the presence of varying concentrations. The rate of Glut-Ala-Ala-Phe-MNA hydrolysis was measured via reverse transcriptase polymerase chain reaction (RT-PCR) using a QIAGEN column (Qiagen) and an RNeasy Mini Kit (Qiagen). Using 5 μg of the harvested RNA, cDNAs were produced with a Superscript First Strand Synthesis kit (Invitrogen) using the oligo(dT) primer included in the kit. Using NEP specific primers (5'-aaatggagctaccga-3' and 5'-ttctgagctgctgcc-3') and primers for β-actin (5'- tagagccagcagtgat-3' and 5'-tttgagctcactcaacc-3') for controls, relative levels of cDNA were measured by comparing product formation at 20, 25, 30, and 35 cycles in PCR reaction comparing under standard conditions using 50°C annealing temperature.

Statistical Analysis

Statistical analysis comparing wild-type NEP and its mutants was performed with Prism 4 software using a two-tailed paired t-test with a 95% confidence interval.

Supporting Information

Figure S1 Sites Mutated in NEP. The active site region of the NEP-phosphoramidon complex [23] is shown with the protein in a ribbon and surface representation and the bound ligand in a stick representation. The mutated residue positions are in red with side chains shown. The zinc ion cofactor is represented by a yellow sphere. Phosphoramidon residues equivalent to substrate peptide positions P1–P2’ are indicated. The approximate position of substrate P2 and P3 residues is shown by the blue ovals. Purple arcs indicate contact between the P1’ residue and F563.

(TIF)

Figure S2 NEP and mutant NEPs produce similar levels of mRNA. Varying PCR cycles were used to estimate the relative amount of NEP mRNA of high and low expressing mutants. Total RNA was harvested from HEK293T cells 96 hrs post transfection and an equal amount of RNA was used as a template for first-strand synthesis to produce a cDNA library using an oligo(dT) universal primer. The cDNA libraries were then used as templates for PCR using primers specific for NEP (experimental) and β-Actin (control). Samples from PCR cycles 20, 25, and 30 were used to estimate NEP transcript levels. The NEP563K mutant product band intensity relative to NEP was 0.9, 1.0, and 1.3 at cycles 20, 25, and 30 respectively. NEP563V and NEP563L were at a level approximately half of the wild-type NEP transcript. In contrast, NEP563L exhibited the same activity as wild-type enzyme while NEP563V displayed ~25% of the wild-type activity, while the activity for NEP563L was undetectable (<1% relative to wild-type enzyme) under our assay conditions (Table 1).

(TIF)

Figure S3 Determination of the concentration of NEP mutants. Purified NEP samples were subjected to SDS-PAGE on 8% polyacrylamide gels and stained for protein with Sypro Ruby dye (A). The gel contained 100, 250, and 500 ng of purified NEP, which was used to construct a standard curve (C) from which the concentration of each NEP form was calculated. Samples of purified NEP, NEP563V, and NEP563L were run at 15 μl and 30 μl. Intensities of each NEP band were fit to the standard curve (C) to give 8.9±0.1, 12.7±3.7, and 11.6±1.1 ng/μl for NEP, NEP563V, and NEP563L, respectively (E, solid bars). Similarly a
Western blot derived from a 10% SDS-PAGE was run containing 10, 50, and 100 ng of purified NEP from which a standard curve was derived (D). NEP, NEP5536E, and NEP5536L were run at 3.75 μL and 7.50 μL. Intensities of each NEP band were fit to the standard curve (D) to give 11.0±1.4, 13.3±0.6, and 13.1±2.2 ng/μL for NEP, NEP5536E, and NEP5536L, respectively (E, empty bars). Note - the difference in size between the NEP standard and the NEP experimental samples is due to differences in glycosylation. Intensities of each NEP band were fit to the standard curve (D) to give 11.0±1.4, 13.3±0.6, and 13.1±2.2 ng/μL for NEP, NEP5536E, and NEP5536L, respectively (E, empty bars). Note - the difference in size between the NEP standard and the NEP experimental samples is due to differences in glycosylation between NEP isolated from CHO cells and HEK cells, respectively.

(TIF)

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Author Contributions

Conceived and designed the experiments: TS DWR LHB LBH. Performed the experiments: TS LH. Analyzed the data: TS DWR LHB LBH. Contributed reagents/materials/analysis tools: TS DWR LHB LBH. Wrote the paper: TS DWR LHB LBH.