SWAPPING THE SUBSTRATE SPECIFICITIES OF THE NEUROPEPTIDASES NEUROLYSIN AND THIMET OLIGOPEPTIDASE*

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Thimet oligopeptidase (EC 3.4.24.15) and neurolysin (EC 3.4.24.16) are closely related zinc dependent metallopeptidases that metabolize small bioactive peptides. They cleave many substrates at the same sites, but they recognize different positions on others, including neurotensin, a 13-residue peptide involved in modulation of dopaminergic circuits, pain perception, and thermoregulation. On the basis of crystal structures and previous mapping studies, four sites (Glu-469/Arg-470, Met-490/Arg-491, His-495/Asn-496, and Arg-498/Thr-499; thimet oligopeptidase residues listed first) in their substrate-binding channels appear positioned to account for differences in specificity. Thimet oligopeptidase mutated so that neurolysin residues are at all four positions cleaves neurotensin at the neurolysin site and the reverse mutations in neurolysin switch hydrolysis to the thimet oligopeptidase site. Using a series of constructs mutated at just three of the sites, it was determined that mutations at only two (Glu-469/Arg-470 and Arg-498/Thr-499) are required to swap specificity, a result that was confirmed by testing the two mutant constructs. If only either one of the two sites is mutated in thimet oligopeptidase, then the enzyme cleaves almost equally at the two hydrolysis positions. Crystal structures of both two mutant constructs show that the mutations do not perturb local structure, but side chain conformations at the Arg-498/Thr-499 position differ from those of the mimicked enzyme. A model for differential recognition of neurotensin based on differences in surface charge distribution in the substrate binding sites is proposed. The model is supported by finding that reducing the positive charge on the peptide results in cleavage at both hydrolysis sites.

Bioactive peptides that serve as signaling molecules in the central nervous system and periphery are inactivated or modified by a group of enzymes known as neuropeptidases. With few exceptions, these enzymes are metallopeptidases that carry out peptide bond hydrolysis with the assistance of a zinc ion cofactor. The neuropeptidases thimet oligopeptidase (TOP¹, EC 3.4.24.15) and neurolysin (EC 3.4.24.16) are closely related members of the zinc metallopeptidase M3 family (1-9). They contain a thermolysin-like catalytic domain that includes a His-Glu-Xaa-Xaa-His (HEXXH) sequence motif involved in zinc ion coordination and catalysis. Both enzymes are widely distributed in mammalian tissues and are found in different subcellular locations (5,10-21) where they primarily metabolize small, bioactive peptides involved in a range of physiological processes. In addition, TOP has been shown to degrade peptides released by the proteasome, limiting the extent of antigen presentation by MHC class I molecules (22-27), and it has been associated with amyloid protein precursor processing (28).

Human neurolysin and TOP share 63% sequence identity over 677 common residues, and the crystal structures of both enzymes (29,30) show that they adopt almost identical folds, with a r.m.s. deviation on Cα atoms of only 1.2 Å. Each
enzyme is split into two domains by a deep, narrow channel that runs the length of the molecule, and the active site is located near the bottom of this groove. The neurolysin/TOP fold is conserved in the M3 family member dipeptidyl carboxypeptidase (31), as well as the more distantly related metallopeptidases angiotensin converting enzyme (ACE) (32), angiotensin converting enzyme related carboxypeptidase (ACE2) (33), and *Pyrococcus furiosus* carboxypeptidase (34). Shielding of the active site by the channel walls in neurolysin and TOP, as well as the structurally related enzymes, restricts them to small peptide substrates (35-40). In addition, these two enzymes share with some other neuropeptidases the ability to recognize highly diverse cleavage site sequences while maintaining specificity for those sites. This fuzzy recognition (41) allows the enzymes to play different roles in different tissues and subcellular locations, and understanding the mechanism underlying this ability is a key objective in the study of neuropeptidases.

Neurolysin and TOP cleave most bioactive peptides at the same site or sites, but they recognize different positions on some naturally occurring and synthetic peptides (1,42-45). Notably, they cleave at distinct sites on the 13-residue bioactive peptide neurotensin, for which they are the primary metabolizing enzymes (4,6,46-51). Neurotensin (NT) modulates central dopaminergic and cholinergic circuits, and it is associated with thermoregulation, intestinal motility, and antinociception (52-55). Neurolysin cleaves the peptide between Pro-10 and Tyr-11, and TOP cleaves between Arg-8 and Arg-9. This difference in site preference represents an opportunity to begin unraveling the basis of substrate recognition in these enzymes.

From their crystal structures and mapping studies, four differences in amino acid sequence were identified as potentially accounting for the differences in NT cleavage (30,56). Here we have mutated these residues in both neurolysin and TOP in order to test their role in mediating differential substrate specificity. We find that by swapping the amino acids at just two of the positions we can swap cleavage sites on the NT peptide, the reengineered neurolysin now cleaving at the wild type TOP site and visa versa. We discuss these and additional results in light of a possible mechanism for differential recognition.

**EXPERIMENTAL PROCEDURES**

**Preparation of TOP and Neurolysin Expression Constructs** – Construction of overexpression vectors for human TOP (pET32 vector, Invitrogen) and rat neurolysin (pBAD vector, Invitrogen) has been described (30,57). Mutagenesis of the coding sequences that produced the substituted proteins was carried out using the QuickChange site-directed mutagenesis kit (Stratagene). Constructs were completely sequenced to confirm the results of the mutagenesis.

**Expression and Purification** – All TOP variants were expressed in *Escherichia coli* BL21(DE3)RP cells and purified as previously described (30). Neurolysin variants were overexpressed in *Escherichia coli* TOP10 cells and purified as reported (57). Purified TOP and TOP mutants were dialyzed against 20mM Tris-HCl (pH 8.0), 5% glycerol and 5mM 2-mercaptoethanol and concentrated to 5-10 mg/ml for storage at 4 °C. Purified neurolysin and neurolysin mutants were dialyzed against 20 mM Tris-HCl (pH 7.4) and 100 mM NaCl and concentrated to 10-15 mg/ml for storage at 4 °C. The level of protein purity was assessed by Coomassie-stained SDS-PAGE (supplemental Fig. S1). In all experiments, the wild type and mutant preparations were found to hydrolyze neurotensin substrate at the known TOP and neurolysin cleavage sites (4,6,46-51) with only low levels of hydrolysis at other sites.

**Determination of primary cleavage sites** – Cleavage site assays were performed with 600 µM NT or NT(R9E) and 0.5 µM of purified proteins in 10 mM HEPES (pH 7.0). NT was obtained from Sigma, Inc. and the NT(R9E) was synthesized by Anaspec, Inc. Reactions were incubated at 37 °C for 10 minutes and then stopped by addition of 0.25% TFA. The hydrolyzed peptides were separated by reverse phase chromatography using a Waters 4.6 x 150 mm C18 column eluted at a flow rate of 1 ml/min with a linear gradient of 10 %-50 % acetonitrile in 0.1 % TFA. Absorbance
was monitored at 214 nm. The products were collected and dried on a centrifugal vacuum evaporator. The fragments’ masses were determined by ESI-TOF mass spectrometry at the Scripps Research Institute Center for Mass Spectrometry.

Kinetic parameters – Analysis of the steady state kinetics for the TOP and neurolysin constructs were performed with 5-6 nM enzyme, 1-6 µM of fluorogenic NT peptide. The fluorogenic peptide (Peptides International) was synthesized with a N-terminal fluorescent 2-aminobenzoyl (Abz) group and a C-terminal quenching ethylenediaminodinitrophenol (EDDnp) group. The hydrolysis reactions were carried out in 25 mM HEPES (pH 7.5), 10 mM NaCl, and 2 mM 2-mercaptoethanol. The fluorescence increase on hydrolysis was followed at 420 nm (excitation at 320 nm) in a LS55 luminescence spectrometer (Perkin Elmer). The change in fluorescence intensity over time was converted into rate of product formation at each substrate concentration. The conversion factor (200 fluorescence units/µmol product) was determined by reactions allowed to proceed to completion. The kinetic parameters were calculated by fitting initial velocity versus substrate concentration to the Michaelis-Menten equation, \( V = \frac{V_{\text{max}} [S]}{K_m + [S]} \), using nonlinear regression (Prism Version 4 software, GraphPad Inc.) (58,59). Errors in \( k_{\text{cat}} \) and \( K_m \) values were estimated based on the deviation of the observed initial velocities from the model (60). High substrate concentrations were not used in determining kinetic parameters because deviations from ideality were observed. Substrate inhibition effects have been reported in angiotensin converting enzyme, another peptidase with the TOP/neurolysin fold (61).

Crystallization of TOP and neurolysin mutants – The TOP2 mutant was crystallized by hanging drop vapor diffusion at 4 °C under conditions similar to those described previously (30). Briefly, 1 µl of 10 mg/ml protein was mixed with 1 µl of well solution containing 100 mM sodium cacodylate (pH 6.5), 100 mM magnesium acetate, 2 mM 2-mercaptoethanol, and 12-14 % (w/v) polyethylene glycol 6000. For data collection, crystals were transferred for a few seconds into a solution containing 25 % glycerol, 100 mM sodium cacodylate (pH 6.5), 100 mM magnesium acetate, 2 mM 2-mercaptoethanol, and 12-14 % (w/v) polyethylene glycol 6000, mounted in a nylon loop (Hampton), and flash cooled by plunging into liquid nitrogen (62). Neurolysin2 mutant crystals were also obtained by hanging drop vapor diffusion at 4 °C following a similar procedure to that reported (57). The well solution for neurolysin was 100 mM sodium cacodylate (pH 6.5), 100 mM magnesium chloride, 0.1 mM zinc chloride, 1 mM 2-mercaptoethanol, and 10-12 % (w/v) polyethylene glycol 8000. The crystals were grown by mixing 1-2 µl of 15 mg/ml protein with an equal volume of well solution. In preparation for data collection, crystals were exposed for a few seconds to a solution containing the same components as the well solution plus 20 % polyethylene glycol 400 and flash cooled as described above.

Data collection and structure determination – X-ray data were collected at the Advanced Photon Source beamline 22-ID (Southeast Regional Collaborative Access Team), Argonne National Laboratory. Data were reduced with HKL2000 (63) and initial structures of TOP2 and neurolysin2 were determined by molecular replacement with the CNS software package (64) using native TOP and neurolysin coordinates, respectively (Protein Data Bank codes 1S4B and 1I1I). Model building and analysis were performed by using the program O (65) and structures were refined with CNS. The space group of the TOP2 crystals is P2 12121 and the cell dimensions are \( a=77.1 \) Å, \( b=99.3 \) Å, \( c=105.7 \) Å. The space group of the neurolysin2 crystals is P2 12 1 2 and the cell dimensions are \( a=159.6 \) Å, \( b=87.7 \) Å, \( c=58.4 \) Å.

RESULTS

Residue Positions Possibly Mediating Differential Recognition – Recognition of different hydrolysis sites on the peptide NT by TOP and neurolysin (Fig. 1A) provides an opportunity to begin exploring the basis for their substrate specificity. As we noted in earlier studies (30,56), only a relatively small number of the sequence differences between the two enzymes map to the interior of the substrate-binding channel, far fewer.
than expected based on its surface area. Of these sequence differences, the four changes identified as being both conserved in orthologs and well positioned to affect substrate specificity are Glu-469/Arg-470, Met-490/Arg-491, His-495/Asn-496, and Arg-498/Thr-499 (TOP residues listed first; Fig. 1B,C). Two of the sites (Glu-469/Arg-470 and Arg-498/Thr-499) are relatively close to the active sites of the enzymes, while the other two are located near one end of the channel where they might interact with N-terminal residues of the NT substrate. We suggested (30) that in the case of neurolysin, Arg-491, Asn-496, and Thr-499 from the enzyme might interact, respectively, with the side chains of Glu-4, Lys-6, and Arg-9 from the peptide to help fix its alignment relative to the active site. In TOP, His-495 and Glu-469 might interact with Glu-4 and Arg-9 from the peptide to help determine the observed cleavage site. The presence of an arginine at position 498, might also play a role in TOP, since with the peptide in the neurolysin registration, that arginine would be positioned opposite Arg-8 and Arg-9 of the peptide, a potentially unfavorable cluster of positive charges.

We began attempts to test the role of the four positions in differential specificity by mutating all the sites in each enzyme to the residues in the corresponding positions of the other enzyme and determining the cleavage site preferences with NT.

Hydrolysis of NT by TOP and Neurolysin Mutants – Cleavage site selection by neurolysin and TOP with the NT substrate peptide were determined by separating reaction fragments using reverse phase HPLC (C18 resin) and identifying individual fragments by electrospray ionization mass spectrometry. As expected, wild type TOP hydrolyzed NT between Arg-8 and Arg-9, producing the fragments NT(1-8) and NT(9-13), while wild type neurolysin cleaved between Pro-10 and Tyr-11, producing fragments NT(1-10) and NT(11-13) (Fig. 2). Experimental and expected masses for these fragments were also the same as those produced by neurolysin, demonstrating that, unlike wild type TOP, the mutant enzyme cleaves primarily between Pro-10 and Tyr-11, mimicking neurolysin site recognition. In the reverse case, the neurolysin four mutant (neurolysin4), produces a hydrolysis pattern indistinguishable from that of wild type TOP, cleaving between Arg-7 and Arg-8. Thus, mutating just the four residues, Glu-469/Arg-470, Met-490/Arg-491, His-495/Asn-496, and Arg-498/Thr-499, is sufficient to completely swap the NT cleavage site recognition of TOP and neurolysin.

Interestingly, wild type neurolysin produces a minor cleavage fragment with the same retention time as the NT(1-8) fragment, and that same minor product is produced by the TOP4 mutant (see Fig. 2). Similarly, wild type TOP produces a fragment or fragments with retention times similar to that of the NT(1-10) fragment, and at least one of these fragments is also produced by neurolysin4. (A minor fragment with a retention slightly less than full length NT is also sometimes seen with neurolysin4.) Not only, then, are the primary cleavage sites swapped by swapping the identities of the four residues, but minor site recognition is also switched.

In order to determine if all four residues must be swapped to switch cleavage site specificity, we produced TOP mutants bearing all the possible three mutation subsets of the four sites first examined (Table 2). Thus each of the TOP constructs have three sites mutated to the amino acids found in neurolysin at the corresponding residue positions. Cleavage site positions for these four TOP mutant constructs, termed TOP3a, TOP3b, TOP3c, and TOP3d, were then determined (Fig. 3A). Two of the mutant constructs, TOP3a and TOP3b, reproduced the cleavage pattern of neurolysin, but the remaining two constructs, TOP3c and TOP3d, cleaved at both the primary site for neurolysin and the
primary site for TOP. This result suggests that both positions, R498T and E469R, that were not mutated in the TOP3c and TOP3d constructs, respectively, are required to alter specificity to that of neurolysin. Either mutation in the absence of the other (in the context of the three mutant constructs) gives cleavage at both the TOP (Arg-6 and Arg-7) and the neurolysin (Pro-10 and Tyr-11) sites (also see supplemental Fig. S2). On the other hand, neither the M490R or H495N mutations, which were not mutated in the TOP3a and TOP3b constructs, respectively, is required to convert TOP to neurolysin specificity. Therefore, only the two residue positions near the active site serve to determine differential specificity. The more remote two sites play no role in differential recognition of NT cleavage positions.

We confirmed this result by making the two double mutants, neurolysin R470E and T499R (neurolysin2) and TOP E469R and R498T (TOP2), and determining their primary hydrolysis sites on NT. As expected, neurolysin2 cleaved at the TOP site, between Arg-6 and Arg-7, while TOP2 cleaved at the neurolysin site, between Pro-10 and Tyr-11 (Fig. 3B). Minor products produced by the wild type enzymes are also produced by the mutant constructs. Thus, altering two of the residues that differ between TOP and neurolysin in the substrate binding channel exchanges cleavage site preference between the two enzymes.

**Kinetics of NT Hydrolysis** – To further characterize the extent of specificity reversal, we measured steady state kinetic parameters for neurolysin hydrolysis by the two double mutant enzymes and compared them to those of wild type neurolysin and TOP (Table 3 and supplemental Fig. S3). The $k_{cat}$ and $K_m$ values of neurolysin2 are very similar to those of TOP, indicating that not only the position but also the kinetic characteristics are swapped by these two mutations. The kinetic parameters of TOP2 are also very similar to those of wild type neurolysin, although the $K_m$ of TOP2 is slightly higher than that of neurolysin. Given the remaining sequence and structural differences between the two enzymes in the binding channel, this relatively small difference in $K_m$ is not unexpected. Otherwise, though, the kinetic parameters of the mutant constructs are the same within error as those of the wild type enzymes being mimicked. Remarkably, altering just two residues in the binding site completely retargets the two enzymes.

We do note, however, that in the cleavage site assays (Figs. 2A, 3A, and 3B), the two mutations that swap specificity appear to increase the degradation rate of NT over the wild type enzymes. These assays are conducted with the unmodified substrate peptide under somewhat different solution conditions from the kinetic assays, which use the fluorogenic peptide substrate. It is therefore possible that some kinetic differences exist between the wild type enzymes and their mutant mimics, depending on the solution conditions and exact nature of the substrate.

**Crystal Structures of TOP2 and Neurolysin2** – Introducing the specificity-altering mutations into TOP and neurolysin might have structural effects beyond simply changing the chemical nature of the side chains. Such effects, if present, would influence interpretation of the reengineering results. The structural effects of introducing the mutations were investigated by determining the crystal structures of the reengineered enzymes. The TOP2 and neurolysin2 mutants were crystallized under similar conditions to those used for wild type TOP and neurolysin, and the crystal structures were determined at 1.94 Å and 2.2 Å, respectively, by molecular replacement with the coordinates of the wild type enzymes (29,30). The refined crystal structure of TOP2 has 654 residues and 471 waters with $R_{work}$ and $R_{free}$ values of 0.202 and 0.238, respectively (Table 4). The neurolysin2 structure has 665 residues and 209 waters with an $R_{work}$ of 0.219 and an $R_{free}$ of 0.268. Electron density maps define clearly the mutant side chains at positions Arg-469/Glu-470 and Thr-498/Arg-499 (Fig. 4A,B).

Superimposing the wild type TOP structure on TOP2 demonstrates that the introduction of the two point mutations does not cause any change in either global or local backbone conformation (Fig. 4C,D). Similarly, no changes in main chain conformation relative to the wild type enzyme are caused by introducing the two mutations in the neurolysin2 construct (Fig.
The absence of any substantial conformational changes accompanying mutation indicates that the changes in specificity for NT cleavage sites are caused by the identities of the substituted side chains rather than any larger scale changes in structure.

In TOP2, the side chain of the introduced Arg-469 adopts a similar conformation to the corresponding residues in wild type neurolysin (Fig. 5A). However, the side chain of the second mutated residue, Thr-498, adopts a different \( \chi_1 \) rotamer from Thr499 in neurolysin (Fig. 5B). It seems likely that this difference in rotamer conformation is due to a structural difference in an adjacent loop segment (Fig. 5C) between TOP and neurolysin (30). The loops (residues 599-611 in TOP and 600-612 in neurolysin) differ in sequence in the two enzymes at a single position, Ala-607 in TOP and Gly-608 in neurolysin. In neurolysin, the loop passes close to Thr-499, and Tyr-606 may be steric contact with the \( \gamma \) methyl of that residue, influencing its conformation. In contrast, the loop in TOP2, which has the same conformation found in wild type TOP, is shifted too far away from Thr-498 to make any contacts. Instead, the side chain of Thr-498 reorients, making hydrogen bond interactions with the side chains of Asn-283 and Gln-608.

For neurolysin2, the comparison with the enzyme being mimicked follows the same pattern. The side chain of the substituted Glu-470 in neurolysin2 has a similar conformation to Glu-469 in wild type TOP (Fig. 5D). Arg-499 adopts a different \( \chi_2 \) rotamer from Arg-498 in TOP, however (Fig. 5E). Again, it seems likely that the differences at position 498/499 are due to differences in the adjacent loop segment (599-611/600-612). In wild type TOP, the side chain of Arg-498 is oriented toward the loop, and in fact makes a hydrogen bond contact with the main chain carbonyl oxygen at Gly-604 (Fig. 5F). This orientation of the arginine side chain would clash with the side chain of Tyr-606 in the shifted loop of neurolysin2, which adopts the same conformation as wild type neurolysin.

The differences in side chain conformation at position 498/499 are interesting in light of our ability of the reengineered enzymes to fully mimic their paralogs. One simple explanation is that the side chains adopt similar conformations when substrate is bound to the enzymes, and differences in binding energetics caused by the variations in initial conformations are small. It is also possible that the side chains do not make direct contacts with the substrate, instead influencing cleavage site selection indirectly, perhaps by altering the electrostatic nature of the binding channel. This possibility is addressed in the Discussion section.

Cleavage of NT(R9E) by TOP and the TOP E469R Mutant – In the published model of NT binding to TOP (30), it was suggested that Glu-469 of the enzyme may form a salt bridge with Arg-9 of NT (Fig. 6A). If such a direct interaction occurs, it seems likely that the interacting residues could be swapped between peptide and enzyme without affecting the salt bridge contact. Therefore, Arg-9 of NT was changed to Glu-9 to produce a peptide referred to as NT(R9E) (Fig. 6B), and TOP Glu-469 was replaced with an arginine. Cleavage site analysis was then performed with the altered peptide and enzyme. Analysis of NT(R9E) cleavage by wild type TOP was done for comparison.

On the basis of modeling studies, we expected that NT(R9E) would be cleaved by the TOP(E469R) mutant between Arg8 and Glu9, the wild type TOP cleavage site. Instead TOP(E469R) cleaved NT(R9E) at two sites, between Arg-8 and Glu-9 and between Pro-10 and Tyr-11 (Fig. 6C, Table 5). This result does not support the formation of a salt bridge between Glu-469 and Arg-9 in the wild type enzyme. Interestingly, wild type TOP also cleaved NT(R9E) at the same two positions. Thus altering the residue at position 9 of NT appears to decrease generally the discrimination between the two sites.

DISCUSSION

Our study is one of a relatively few examples of reengineering substrate specificity in peptidases (66-69). Mapping sequence differences between enzymes based on their structural models (29,30) proved a successful way of identifying residues that determine differences in specificity between these two closely related enzymes. It has
not always been possible, however, to completely swap specificities between related enzymes despite extensive structural knowledge and considerable effort (66). For example, there have been a number of attempts to convert specificity between trypsin and chymotrypsin by substituting amino acids in multiple positions of the protein, including exchange of four residues in the S1 site and six residues in two surface loops which do not directly contact the substrate (70-72). The best reengineered trypsin mutant does indeed hydrolyze substrates containing large hydrophobic residues at the P1 position, like chymotrypsin, but its substrate affinity is greatly reduced (70).

Only two (Glu-469/Arg-470 and Arg-498/Thr-499) of the four identified residue differences between TOP and neurolysin proved to actually mediate differential specificity with respect to NT. The two residue positions that did not affect specificity (Met-490/Arg-491 and His-495/Asn-496) are located at one end of the channel far from the active site (see Fig. 1). Models of NT binding to neurolysin and TOP (29,30) suggest that the N terminus of the peptide would bind at this end of the channel, which is consistent with considerable evidence from structural studies of other peptidases with thermolysin-like active sites (31,73-76). The results of this study suggest that either this model is incorrect or that the peptide does not interact strongly with residues at the mutated positions despite their proximity.

Recent work indicates that initial recognition of peptide substrates by TOP and neurolysin is mediated by an unusual surface located across the channel from the active site. The surface, which is largely conserved in neurolysin, interacts with peptide residues C terminal to the scissile bond in a number of cocrystal structures with TOP. In the TOP-NT complex crystal structure, the residues from P2 to the N terminus of the peptide are disordered, suggesting that they do not play a significant role in initial binding. This possibility is consistent with the lack of a role for the distant residues in differential specificity. Interestingly, the TOP-NT crystal structure also shows that, in contrast to earlier models for neurotensin binding (29,30), the peptide does not interact directly with either of the two residue positions shown here to mediate differential specificity. One corresponding position in neurolysin, Arg-470, is also unlikely to contact bound NT, assuming binding similar (but shifted in registration) to that seen for TOP (although a possible interaction with the C-terminal carboxylate of NT cannot be completely ruled out). While the role of other residue, Thr-499, in the neurolysin-NT interaction remains unknown in the absence of a structure for the complex, the different side chain conformations at this position in neurolysin and TOP (29,30) provide some support for the absence of a direct contact between this residue and NT as well.

If the residues at the two critical positions do not directly interact with NT, how then do they cause TOP and neurolysin to cleave the substrate peptide at different sites? A difference in surface electrostatic potential in TOP and neurolysin at these positions suggests a possible model. In TOP, the presence of Glu-469 near the open end of the channel and Arg498 on the other side of the active site helps to create a strong gradient in electrostatic potential at the bottom of the substrate binding channel (Fig. 7A). A concentration of negative potential near the open end of the channel is particularly prominent. In neurolysin on the other hand, the substitutions of Arg-470 for Glu-469 and Thr-499 for Arg-498 are largely responsible for reducing or even reversing this gradient. NT clearly has two sites that interact with relatively high affinity with the primary substrate binding surfaces of TOP and neurolysin described above. Cleavage at both peptide sites (Arg-8-Arg-9 and Pro-10-Tyr-11) by the engineered enzymes bearing only one of the two critical mutations (see Fig. 3) and the fact that even the wild type enzymes cleave slowly at the primary site of the other enzyme (see Fig. 2, for example) are evidence for two strongly interacting sequences in the substrate. Since NT has a highly basic region (Lys-6-Pro-7-Arg-8-Arg-9) near the center of its sequence, it is possible that the overall electrostatics of the binding site influences which of the two cleavage sites interacts with the binding surfaces of the enzymes. In this model (Fig. 7B), the strong electrostatic gradient in TOP, with the highly negative surface near the open end of the channel, would favor the cleavage site (Arg-8-Arg-9) that places the positive region of the
peptide closer to the locus of negative charge on the enzyme. The reversed gradient in neurolysin, in contrast, would favor the cleavage site (Pro-10-Tyr-11) that shifts the positive region of NT further from the open end of the channel. Substitutions at the two critical positions therefore act as an electrostatic switch, shifting the registration of NT between the two sites that interact strongly with the primary binding surface. Bioactive peptides that TOP and neurolysin cleave at the same site (1,42-45) would have only one cleavage sequence that interacts strongly with the binding site on the enzyme. In that case, differences in the overall electrostatic potential at the channel floor would not be sufficient to cause different binding registrations in the two enzymes. Also, peptides that do not have a region of concentrated electrostatic potential would not be greatly influenced by surface charge gradients in the binding channels, allowing the enzymes to cleave a the same single or multiple sites that interact with high affinity. An electrostatic difference between the two subunits of the metallopeptidase meprin has been shown recently to be important in determining differences in substrate preference (69), and general electrostatic effects are believed to be important in the substrate recognition by other peptidases (77-79).

This model for differential recognition is consistent with the results of the experiment in which glutamate and arginine residues were swapped between the peptide and enzyme (Fig. 7C; see Fig. 6). Changing Arg-9 in NT to a glutamate to make the NT(R9E) peptide reduces the formal charge in the central region of the substrate, making it less susceptible to steering by the electrostatic gradient present in TOP. As expected, wild type TOP then cleaves at both strong binding sites of this altered peptide. (The change to glutamate in NT(R9E) does not seem to alter the number of strongly interacting sites.) Decreasing the strength of the positively charged region of the peptide is apparently sufficient to completely negate the electrostatic steering effect, since the TOP(E469R) mutant, which has a reduced electrostatic gradient, behaves like wild type with respect to cleavage of NT(R9E). With the unaltered NT peptide, though, the tests with the three mutant constructs (see Fig. 3), as well as the single mutant TOP(E469R) (see Fig. S2), demonstrate that the E469R mutation in TOP is itself also sufficient to give hydrolysis at the two positions. The similar outcomes when either the electrostatic gradient on the enzyme surface or the charge on the peptide is reduced argue strongly for the electrostatic steering model.

The results reported here suggest that the two positions mediating differential recognition with respect to NT do not play a significant role in maintaining the broad specificity of TOP and neurolysin. Instead, the fuzzy recognition is likely to arise largely from the characteristics of the primary binding surface. This work does indicate, however, that it is possible to modulate the primary hydrolysis site by affecting the overall electrostatic properties of the substrate-binding channel. In that regard, other positions beside the two identified could be used to switch between the two NT cleavage sites. The ability to produce the same switching effect by mutating other sites, particularly for sites even more unlikely to interact directly with substrate, would provide additional support for an electrostatics-based model. Modulation of the substrate hydrolysis site seen here is probably one aspect of a more general electrostatic steering of substrate into the deep, narrow binding channels of TOP and neurolysin, since the walls of the channels carry an overall negative charge (30,56). The importance of this steering effect for substrate binding remains to be explored in detail, however.

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**FOOTNOTES**

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The atomic coordinates and structure factors (thimet oligopeptidase two mutant, code 2O36; neurolysin two mutant, code 2O3E) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: TOP, thimet oligopeptidase; NT, neurotensin, NT(R9E), neurotensin modified peptide (Arg9Glu); TOP4, thimet oligopeptidase mutated to the neurolysin sequence at four residue positions; neurolysin4, neurolysin mutated to the thimet oligopeptidase sequence at four residue positions; TOP2, thimet oligopeptidase mutated to the neurolysin sequence at two residue positions; neurolysin2, neurolysin mutated to the thimet oligopeptidase sequence at two residue positions.

2 Nicholas Noinaj and David W. Rodgers, unpublished observations.

**FIGURE LEGENDS**

Fig. 1 Neurotensin (NT) cleavage sites and key residue differences in the substrate binding channels of TOP and neurolysin. A, The sequence of the peptide neurotensin is shown, and the primary hydrolysis sites for the enzymes TOP and neurolysin are indicated. B, Cut away molecular surface view of the TOP substrate-binding channel with residues likely to mediate differences with neurolysin in neurotensin cleavage site. C, Same view of the neurolysin substrate-binding channel with corresponding residues indicated. In both cut away views, the active site zinc ion is shown as a blue sphere.

Fig. 2. Cleavage of NT by the TOP4 and neurolysin4 mutants and the wild type enzymes. A, Reverse phase HPLC separation of hydrolysis products generated by the enzymes indicated for each absorption trace. The traces are offset in the vertical direction for clarity. Peaks were identified by mass spectrometry determination of molecular mass. The numbered peaks are: 1, NT(1-8); 2, NT(1-10); 3, NT(9-13); 4, NT(11-13); 5, full length NT. B, Diagram showing schematically the NT cleavage fragments and associated peak numbers.

Fig. 3. Cleavage of NT by the TOP3 mutants, the TOP2 and neurolysin2 mutants, and the wild type enzymes. A, Reverse phase HPLC separation of hydrolysis products by the TOP3a-d mutants and the wild type enzymes. Absorption traces are offset in the vertical direction for clarity, and the enzyme used is indicated for each trace. Hydrolysis fragments are assigned the same peak numbers used in Fig. 2. B, Separation of cleavage products resulting from cleavage
with the TOP2 and neurolysin2 mutants and the wild type enzymes. Peaks are numbered as in Fig. 2.

Fig. 4. Crystal structures of TOP2 and neurolysin2. A, Electron density (2Fo-Fc) for the substituted side chains in the TOP2 mutant. Density is shown as in wire-frame representation (green) at the r.m.s.d. of the map. B, Electron density for the substituted side chains in neurolysin2 shown at the r.m.s.d. of the map. C, Backbone worm representation of superimposed wild type TOP and TOP2. D, Close up of superimposed wild type TOP and TOP2 showing both positions substituted in TOP2 and the active site. E, Backbone worm representation of superimposed wild type neurolysin and neurolysin2. F, Close up of superimposed wild type neurolysin and neurolysin2 showing both positions substituted in neurolysin2 and the active site.

Fig. 5. Comparison of the substituted residues in TOP2 and neurolysin2 with the corresponding residues in wild type neurolysin and TOP. A, The side chain of Arg-469 from TOP2 (cyan) with Arg-470 from wild type neurolysin (green). Backbone atoms are shown in a worm representation. B, Thr-498 from TOP2 (cyan) and Thr-499 from neurolysin (green). C, Conformational differences between TOP2 (cyan) and neurolysin (green) in a loop near the Thr498/499 position. In addition to Thr-498/499, residues 599-611 of TOP2 and residues 600-612 of neurolysin are shown in a backbone worm representation. The side chains of Tyr-605/606 in the two structures are also shown. D, Glu-470 from neurolysin2 (green) and Glu-469 from wild type neurolysin (cyan). E, Arg-499 from neurolysin2 and Arg-498 from wild type TOP. F, Loop region in neurolysin 2 (green) and wild type TOP (cyan) near Arg-499/498. Residue ranges are the same as in panel C.

Fig. 6. Cleavage of NT(R9E) by TOP(E469R) and wild type TOP. A, Possible salt bridge between Glu-469 from TOP and Arg-9 from NT is modeled (30,56). The active site helix and zinc ion coordinating residues of TOP are shown along with the side chain of Glu-469. Backbone atoms of modeled NT are shown in a stick representation with the side chain of Arg-9. The cleavage site for TOP is indicated. B, Reverse phase HPLC separation of a synthetic NT(R9E) substituted peptide hydrolysis fragments after incubated with the indicated enzymes. Absorption traces are offset vertically for clarity. Peak numbers refer to product fragments identified by mass spectrometry (see panel C). C, Sequence of the NT(R9E) peptide and schematic representation of hydrolysis fragments, which are assigned numbers followed by a letter to distinguish them from NT cleavage fragments.

Fig. 7. Model for differential specificity of TOP and neurolysin with respect to primary NT hydrolysis sites. A, Cut away molecular surface views of the TOP (left) and neurolysin (right) binding sites colored according to surface electrostatic potential (red, negative; blue, positive). The active site zinc ion is shown as a blue sphere. Schematic representations of the NT peptide in two binding registrations emphasizing the positively charged region in the center of the peptide are also shown. B, Schematic NT representations with the key residues mediating differential specificity shown along with their contribution to surface electrostatic potential in the substrate binding site. C, Similar representation of the NT(R9E) peptide with the key residues in wild type TOP and the TOP(E469R) mutant.

Fig. S1. Purity of TOP and neurolysin constructs. A representative Coomassie-stained SDS-polyacrylamide gel (12%) is shown for the indicated constructs. Molecular weight markers are also shown with their sizes in kDa indicated on the left.

Fig. S2. Cleavage of neurotensin by the TOP(E469R) mutant. Absorption traces for reverse phase HPLC separation of neurotensin hydrolysis products generated by TOP, TOP(E469R), and
neurolysin are shown. The labeled traces are offset in the vertical direction for clarity. Peaks were identified by mass spectrometry determination of molecular mass. The numbered peaks are: 1, NT(1-8); 2, NT(1-10); 3, NT(9-13); 4, NT(11-13); 5, full length NT.

Fig. S3. **Kinetics of fluorogenic NT hydrolysis by TOP, TOP2, neurolysin, and neurolysin2.** Plots of initial velocity versus substrate concentration are shown for the hydrolysis of Abz-NT-EDDnp by: A, wild type TOP; B, TOP2; C, wild type neurolysin; and D, neurolysin2. The curves show the nonlinear regression fits of the Michaelis-Menten equation to the data. These fits were used to determine the kinetic parameters and their error estimates.
TABLE 1. Molecular masses$^a$ of NT fragments produced by wild type TOP, wild type neurolysin, and TOP4

| Enzyme      | NT1-8 (expected mass) | NT1-10 (expected mass) | NT9-13 (expected mass) | NT11-13 (expected mass) | NT1-13 (expected mass) |
|-------------|-----------------------|------------------------|------------------------|------------------------|------------------------|
| Wild type   | 1030.5                | -                      | 661.4                  | -                      | 1672                   |
| TOP         | (1030.1)              | (660.8)                |                        |                        | (1672.9)               |
| Wild type   | -                     | 1283.6                 | -                      | 408.2                  | 1672                   |
| Neurolysin  | (1283.4)              | (407.5)                |                        |                        | (1672.9)               |
| TOP4        | -                     | 1283.6                 | -                      | 408.2                  | -                      |
|             | (1283.4)              | (407.5)                |                        |                        |                        |

$^a$ Masses were determined by ESI-TOF mass spectrometry and are given in Daltons.
TABLE 2. Summary of mutation sites and NT hydrolysis by the TOP3 mutants

| Mutations Introduced<sup>a</sup> | TOP3a mutant | TOP3b mutant | TOP3c mutant | TOP3d mutant |
|-------------------------------|--------------|--------------|--------------|--------------|
| mutations                     |              |              |              |              |
| M490R                         | -            | x            | x            | x            |
| H495N                         | x            | -            | x            | x            |
| R498T                         | x            | x            | -            | x            |
| E469R                         | x            | x            | x            | -            |

Cleavage pattern

| Cleavage pattern of NT | Neurolysin site<sup>a</sup> | Neurolysin site | Neurolysin and TOP sites<sup>b</sup> | Neurolysin and TOP sites |
|------------------------|-----------------------------|-----------------|--------------------------------------|--------------------------|
| Cleavage pattern of NT | site<sup>a</sup>            | site            | TOP sites                            | TOP sites                |

<sup>a</sup> The symbol x indicates that the mutation was present in the construct. The mutations introduce into thimet oligopeptidase the amino acid found in neurolysin at the equivalent residue position.

<sup>b</sup> Hydrolysis between Pro10 and Tyr11.

<sup>c</sup> Hydrolysis at two positions, Arg8-Arg9 and Pro10-Tyr11.
**TABLE 3.** Kinetics parameters for hydrolysis of fluorogenic NT by wild type TOP, TOP2 mutant, wild type neurolysin, and neurolysin2 mutant

| Enzymes            | $k_{cat}$ (sec$^{-1}$) | $K_m$ (µM)   |
|--------------------|------------------------|--------------|
| Wild type TOP      | 2.3 ± 0.2              | 2.6 ± 0.3    |
| Neurolysin2 mutant | 2.79 ± 0.42            | 2.95 ± 0.45  |
| Wild type Neurolysin | 5.0 ± 0.4              | 2.0 ± 0.2    |
| TOP2 mutant        | 4.2 ± 0.65             | 3.3 ± 0.5    |
**TABLE 4.** Summary of crystallographic data and refinement for TOP2 and neurolysin2

|                          | TOP2                  | Neurolysin2            |
|--------------------------|-----------------------|------------------------|
| **Crystallographic data**|                       |                        |
| Wavelength (Å)           | 0.9997                | 0.9997                 |
| Resolution (Å)           | 30-1.94               | 50-2.2                 |
| Last shell (Å)           | 2.01-1.94             | 2.28-2.2               |
| Average redundancy (last shell) (%) | 4.8 (4.4)          | 4.3 (4.2)              |
| \( R_{	ext{sym}} \) (last shell) (%) | 0.075 (0.299)       | 0.088 (0.412)          |
| \( I/\sigma I \) (last shell) (%) | 20.06 (4.68)        | 14.96 (3.98)           |
| Completeness (last shell) (%) | 98.2 (90.5)        | 98.7 (99.6)            |
| **Refinement**           |                       |                        |
| Resolution (Å)           | 30-1.94               | 50-2.2                 |
| Number of reflections included in refinement | 59,387               | 41,630                 |
| \( R_{\text{work}}/R_{\text{free}} \) | 0.202/0.238         | 0.219/0.268            |
| r.m.s.d. bond lengths (Å) | 0.004                | 0.006                  |
| r.m.s.d. bond angles (°) | 1.1                   | 1.2                    |
| r.m.s.d. improper angles (°) | 0.7                  | 0.8                    |
| r.m.s.d. dihedral angles (°) | 19.4                 | 20.2                   |
| B \(^a\) r.m.s.d. bonded atoms (main/side) | 1.4/2.3              | 1.3/2.2                |
| Average B for all protein atoms (Å\(^2\)) | 25.2                 | 39.1                   |
| Average B for ordered solvent (Å\(^2\)) | 30.8                 | 38.2                   |
| Number of solvent molecules | 471                 | 209                    |
| Number of metal ions     | 1                     | 2                      |

\(^a\) Isotropic thermal factor
TABLE 5. Molecular masses$^a$ of NT(R9E) fragments and full length of NT(R9E) by the TOP(E469R) mutant

| Enzyme                | NT(R9E) 1-8 (expected mass) | NT(R9E) 1-10 (expected mass) | NT(R9E) 9-13 (expected mass) | NT(R9E) 11-13 (expected mass) | NT(R9E) 1-13 (expected mass) |
|-----------------------|------------------------------|------------------------------|------------------------------|------------------------------|------------------------------|
| TOP E469R mutant      | 1030.5 (1030.2)              | 1256.66 (1256.4)             | 408.2 (407.5)                | 634.3 (633.7)                | 1646 (1646.8)                |

$^a$Masses were determined by ESI-TOF mass spectrometry and are given in Daltons.
Figure 1

A

TOP cleavage site

pGlu1 – Leu2 – Tyr3 – Glu4 – Asn5 – Lys6 – Pro7 – Arg8 – Arg9 – Pro10 – Tyr11 – Ile12 – Leu13

Neurolysin cleavage site

B

C

Arg491

Asn496

Thr499

Arg470

Zn²⁺
Figure 2

A

B

pGlu1 – Leu2 – Tyr3 – Glu4 – Asn5 – Lys6 – Pro7 – Arg8 – Arg9 – Pro10 – Tyr11 – Ile12 – Leu13

5, NT1-13
1, NT1-8
2, NT1-10
3, NT9-13
4, NT11-13
Figure 3
Figure 4
Figure 5
Figure 6

A

C

Expected TOP(E469R) cleavage site

pGlu1 - Leu2 - Tyr3 - Glu4 - Asn5 - Lys6 - Pro7 - Arg8 - Glu9 - Pro10 - Tyr11 - Ile12 - Leu13

5a, NT(R9E)1-13
1a, NT(R9E)1-8
2a, NT(R9E)1-10

4a, NT(R9E)9-13
3a, NT(R9E)11-13
Figure 7
Figure S1
Figure S2
Figure S3

A

B

C

D

V(µmol/sec) vs. S(µM)

V(µmol/sec) vs. S(µM)
Swapping the substrate specificities of the neuropeptidases neurolysin and thimet oligopeptidase
Eun Jeong Lim, Sowmya Sampath, Jerry Coll-Rodriguez, Jack Schmidt, Kallol Ray and David W. Rodgers

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