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Original Scientific Article

Yeast Ortholog of Peptidase Family M49: the Role of Invariant Glu^{461} and Tyr^{327} †

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Abstract. Metallopeptidase family M49 is characterized by five conserved sequence regions and the unique motif HEXXGH with two histidines - ligands of the active-site zinc ion. The crystal structure of the yeast ortholog represents a prototype for the whole family.

To investigate the role of two invariant amino acid residues, a Glu^{461} of the zinc-binding motif, and a Tyr^{327}, 21Å from the catalytic zinc center, mutational analysis of the yeast enzyme was performed. The substitution of Glu^{461} to glutamine decreased k_{cat} for the substrate hydrolysis almost by 10 000-fold. The replacement of Tyr^{327} by Phe or Ala reduced the catalytic efficiency (k_{cat}/K_m) by two orders of magnitude. The affinity for the heptapeptide valorphin was siginificantly lowered in all mutants, indicating the contribution of both Glu^{461} and Tyr^{327} in substrate binding. Taken together, the effect of mutating Glu^{461} is consistent with this residue being essential in M49 peptidase catalysis. (doi: 10.5562/cca2107)

Keywords: enzyme catalysis, metallopeptidase, protein structure-function, site-directed mutagenesis, yeast *Saccharomyces cerevisiae*

INTRODUCTION

Hydrolysis of peptide bonds is essential for many biological processes. Proteolytic enzymes (peptidases, proteases) catalyze this reaction *in vivo*. Their physiological importance is reflected by the fact that ~2 % of the human genome encodes for peptidases.[1] One of the four main peptidase classes are metallopeptidases, which are mostly zinc-dependent peptide-bond hydrolases.[2] There are several classifications of metallopeptidases, with the one provided by the MEROPS database (http://merops.sanger.ac.uk) being perhaps the most accepted.[3] This classification is based on the catalytic type and amino acid sequence similarity through which different families are recognized.

Metallopeptidase family M49 (also known as peptidase family M49, and dipeptidyl peptidase III family) is characterized by 5 conserved amino acid sequence regions and the unique hexapeptide linear motif HEXXGH which harbors two histidines - ligands of the active-site zinc ion.[4] Members of this family participate in intracellular peptide metabolism. The breakthrough in the research of M49 peptidases was the elucidation of the crystal structure of the yeast orthologue in 2008, which revealed a two-domain protein with a new fold and represented a prototype for this family of proteases.[5] Since then, the investigation of the structure-function relationship of these metallopeptidases accelerated. Most of the work has been performed in the human ortholog by using site-directed mutagenesis, X-ray crystallography and computational approaches, often in combination.[6–8]

Much of the effort was directed towards defining the substrate binding site. Most recently, the first crystal structure of human M49 peptidase (dipeptidyl peptidase III, DPP III) in complex with the pentapeptide tynorphin was reported.[8] This study discovered an exceptionally large domain motion upon ligand binding and confirmed some previous site-directed mutagenesis data which indicated the (functional) importance of conserved amino acid residues from both protein domains for the enzyme activity.[7,9] Meantime, there are still a number of unanswered questions regarding the catalytic mechanism of metallopeptidases of M49 family. One of them is the role of the evolutionary conserved glutamic acid residue which is part of the HEXXGH motif

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(Glu^{451} in human and rat DPP III, Glu^{461} in yeast ortholog). Previous mutational analysis of the rat DPP III showed that Glu^{451} is essential for catalysis and not important for the zinc binding.[10] Molecular dynamics simulations of human DPP III indicated the interactions of Glu^{451} with substrate and inhibitor.[6] However, the experimental evidence for that is missing, since the only known crystal structure of the enzyme-ligand complex was elucidated for the E451A inactive mutant of the human ortholog.[8]

There is also controversy regarding the function of the only conserved tyrosine residue in M49 peptidases (at the sequence position 318 in human, and at position 327 in yeast peptidase). Mutational analysis of the human enzyme implied the importance of Tyr^{318} in transition state stabilization, but not in the small ligand binding (hydroxamate inihibitor Tyr-Phe-NHOH), while the X-ray crystallography pointed to the interaction of this residue with the pentapeptide ligand tynorphin.[8,9]

In order to investigate the role of these two invariant amino acid residues of the M49 family peptidases, Glu^{461} from the active-site motif HEXXGH, and Tyr^{327} from the conserved region 1 of the yeast ortholog,[4,9] was replaced by Gln, and Phe as well as Ala, respectively, by site-directed mutagenesis, and the catalytic properties of generated purified enzyme variants were examined.

EXPERIMENTAL

Cloning and Site-directed Mutagenesis

Yeast DPP III protein with a C-terminal hexa-histidine affinity tag was obtained by PCR amplification of the YOL057W gene using genomic DNA isolated from *Saccharomyces cerevisiae* (BY4741), and cloning into pET21a vector into the *NdeI*/*XhoI* restriction sites as reported previously by Jajčanin-Jozić *et al.*[11]

Point mutations of the DPP III gene E461Q, Y327A and Y327F were carried out with QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, USA) by using previously constructed vector (pET21a-DPPIII6_{His}) as a template, and primers listed in Table 1. All primers were custom synthesized by Invitrogen (USA) or by MWG Biotech AG (Ebersberg, Germany). DNA sequences of cloned constructs, as well confirmation of each mutant were obtained with automated sequence analyzer "ABI PRISM® 3100-Avant Genetic Analyzer" (Applied Biosystem, USA) and also by sequence analysis (MWG Biotech), using T7 forward and T7 reverse primers, as well as the internal DPP III primers: 5'-CATCAACCACTTTGTCACTGG-3' and 5'-CGATATAAAACTTGGCGGATGC-3'.

Heterologous Expression and Purification of Recombinant Proteins

Wild-type His_6-tagged DPP III as well as the three single variants E461Q, Y327A and Y327F were expressed in *Escherichia coli*, and purified employing affinity chromatography on nickel-nitrilotriacetic acid resin (Ni-NTA Agarose, Qiagen) as described earlier.[11]

Protein purity was confirmed by SDS-PAGE carried out in T = 12.5 % polyacrylamide gel according to method of Laemmli[12] and enzymatic activity was assayed using Arg_2-2NA as substrate. All fractions of high purity were pooled and desalted on PD-10 columns (GE Healthcare) equilibrated with 20 mmol dm^{-3} Tris-HCl buffer, pH = 7.4. Purified protein preparations were stored in 15 mmol dm^{-3} Tris-HCl buffer, pH = 7.4 containing 22 % glycerol at –10 °C.

Protein concentrations were determined using the method of Bradford, with bovine serum albumin as a standard[13] and/or by measuring the absorbance at 280 nm using the predicted extinction coefficients.

Enzyme Activity Assay and Kinetic Analysis

The enzymatic activity of yeast wild-type and mutant DPP III were determined spectrophotometrically by a standard assay at 37 °C with Arg_2-2NA as a substrate as described earlier.[11] Kinetic parameters for hydrolysis of Arg_2-2NA were determined fluorometrically at 25 °C at pH = 8.0 in the presence of 100 μmol dm^{-3} solution of $CoCl_2$ by initial rate measurements. The kinetic parameters were calculated using a Hanes plot.[11]

Affinities of wild-type and mutant DPP III for peptide valorphin (K_i values) were determined according to Chu and Orlowski.[14] Hydrolysis of 15 μmol dm^{-3} Arg_2-2NA in 20 mmol dm^{-3} Tris-HCl buffer, pH = 8.0 in the presence and absence of different concentrations of peptide was followed fluorimetrically at 25 °C. K_i was calculated using the reaction rate at peptide concentration which caused inhibition close to 50 %, by the equation $K_i = [I]/(v_o/v_i - 1) \times K_m/(K_m + [S])$.

Table 1. Primers used for site-directed mutagenesis of yeast M49 peptidase DNA

Mutant	Nucleotide sequence (5'–3') Primer I	Nucleotide sequence (5'–3') Primer II
Y327A	cggttttatcgaaaca**gct**agagaaccctcgggc	gcccgagggttctct**agc**tgtttcgataaaaccg
Y327F	cggttttatcgaaaca**ttt**agagaaccctcgggc	gcccgagggttctct**aaa**tgtttcgataaaaccg
E461Q	ccaagtaggcatccat**caa**ttattaggacatggttcagg	cctgaaccatgtcctaataa**ttg**atggatgcctacttgg

Codons for the mutated residues are bolded.

Circular Dichroism Analysis

The far-UV CD spectra were recorded on a JASCO J-815 spectropolarimeter at 25 °C from 190–250 nm, using a quartz cuvette of 0.1 mm path length as described earlier.[11]

The protein concentration used for CD measurements was 0.4 mg ml^{-1}.

RESULTS AND DISCUSSION

Expression, Purification and Kinetic Characterization of Yeast M49 Peptidase and Its Mutants

Recombinant yeast *Saccharomyces cerevisiae* metallopeptidase, wild-type and variants, was purifed by metal affinity chromatography as His-tagged proteins (Figure 1). Previously, we investigated the influence of His_6-tag at the C-terminus of the yeast M49 peptidase on the wild-type enzyme activity and did not find any significant change in kinetic parameters.[11]

Replacement of Glutamate 461 to Glutamine

The exchange of Glu^{461} to glutamine resulted in a dramatic reduction (9200-fold) in the k_{cat} for the hydrolysis of the preferred synthetic substrate Arg-Arg-2-naphthylamide (Arg_2-2NA) and 2.8-fold increase in K_m value (Table 2).

The effect of the mutation on the protein structure was examined by circular dichroism (CD) analysis. The CD spectra of the wild-type and the E461Q variant were nearly identical, indicating that no change in secondary structure is caused by the Glu^{461} to glutamine substitution (Figure 2).

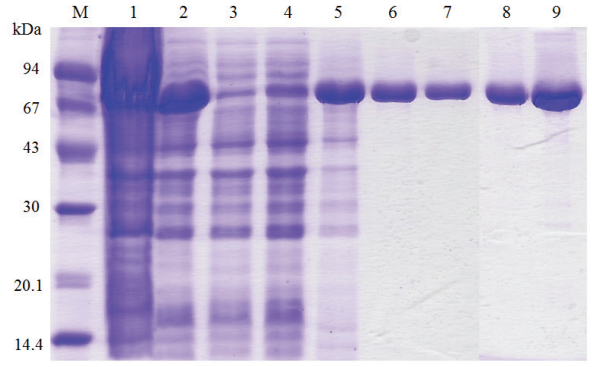

Figure 1. SDS-PAGE (12.5 % gel) analysis of the purification progess of yeast M49 peptidase-His_6 protein (E461Q mutant) by affinity chromatography on Ni-NTA agarose column. M – protein molecular mass standards; 1 – pellet; 2 - *E.coli* cleared cell lysate containing recombinant M49 peptidase protein; 3-4 – flow-through Ni-NTA column; 5-7 – Ni-NTA column wash steps with 20 mmol dm^{-3} imidazole; 8-9 – yeast M49 peptidase-His_6 protein (E461Q mutant) eluted with 150 mmol dm^{-3} imidazole. Proteins were visualized by Coomassie Blue staining.

Table 2. Kinetic characterization (means ± S.E. for three separate determinations) of wild-type yeast M49 peptidase and its mutants

Enzyme form	Arg-Arg-2NA hydrolysis	
	K_m / μmol dm^{-3}	k_{cat} / min^{-1}
Wild-type	15.18 ± 3.03	14.916 ± 3.36
E461Q	42.13 ± 4.05	$0.00162 \pm 4.14 \times 10^{-4}$
Y327A	33.54 ± 2.25	0.072 ± 0.012
Y327F	19.23 ± 0.11	0.108 ± 0.004

The kinetic parameters K_m and k_{cat} were determined from the initial reaction rates at 25 °C and pH = 8.0 with the Arg-Arg-2NA, in the presence of 100 μmol dm^{-3} solution of $CoCl_2$, using a Hanes plot.

Fukasawa *et al.* replaced Glu^{451}, which is a structural equivalent of yeast M49 peptidase Glu^{461}, with an alanine or an aspartic acid residue in the rat DPP III (M49 peptidase).[10] These authors reported that the zinc content was unchanged in their variants (E451A and E451D), compared to wild-type, however, enzyme activity was not detectable, and hence kinetic parameters were not determined. Our present kinetic analysis of the yeast E461Q variant is thus the first quantitative study of this replacement in the M49 family.

Metallopeptidases represent the most diverse of the catalytic types of peptidases, with more than 60 families (MEROPS). The majority of metallopeptidase families comprise enzymes containing the pentapeptide motif HEXXH at their active sites in which the two His are zinc ligands and the Glu has catalytic function (Table 3). In the M49 family, the distance between the zinc-ligating histidines is extended by one residue, and peptidases of family M16 contain the HXXEH motif, an inversion of the more usual active-site HEXXH motif (Table 3).

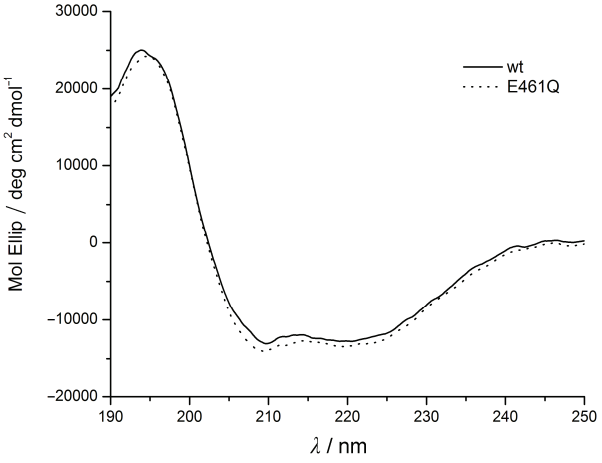

Figure 2. Circular dichroism spectra of wild-type yeast peptidase M49 and E461Q mutant.

Table 3. Alignment of the zinc binding motifs of different zinc metallopeptidases. APA: aminopeptidase A (EC 3.4.11.7), APN: aminopeptidase N (EC 3.4.11.2), PSA: puromycin-sensitive aminopeptidase (EC 3.4.11.14), LTA4H: leukotriene A4 hydrolase (EC 3.3.2.6), TLN: thermolysin (EC 3.4.24.27), NEP: neprilysin (EC 3.4.24.11), DPP III: dipeptidyl peptidase III (3.4.14.4), IDE: insulysin, insulin-degrading enzyme (EC 3.4.24.56). Bold black letters represent zinc-ligating histidines; glutamic acid residue involved in catalysis is underlined in red.

Enzyme	(family)	Zinc binding motif
APA	(M1)	VA**H**ELV**H**QW
APN	(M1)	IA**H**ELA**H**QW
PSA	(M1)	VG**H**ELA**H**QW
LTA4H	(M1)	IA**H**EIS**H**SW
TLN	(M4)	VA**H**ELT**H**AV
NEP	(M13)	IG**H**EIT**H**GF
DPP III	(M49)	GI**H**ELLG**H**GS
IDE	(M16)	LS**H**FCE**H**ML

The importance of the glutamic acid residue in the active-site motif harboring two histidines has been investigated and confirmed by the site-directed mutagenesis in a number of zinc-dependent exo-and endopeptidases. In most cases, the effect replacing Glu in the active site were not quantified. In some cases, the same amount of recombinant wild-type enzyme and variant was used in the assay to compare their activities,[15] while actually a much higher concentration of the variant is required to measure residual enzyme activity. Thompson *et al.* have exchanged the active-site glutamate (Glu^{309}) of the puromycin-sensitive aminopeptidase, a member of family M1, to glutamine, alanine and valine, and succeeded to characterize the variants kinetically, with amino acid β-naphthylamides as substrates.[16] The E309Q variant showed 5700-fold to 16,000-fold reduction in the k_{cat} values, which is comparable with the effect of replacing Glu^{461} to glutamine in yeast M49 peptidase (9200-fold reduction in the k_{cat} for Arg-Arg-β-naphthylamide substrate). However, these authors did not observe significant change in K_m values.[16]

Replacement of Tyrosine 327 to Phenylalanine and Alanine

The replacement of Tyr^{327} decreased the k_{cat} of the yeast M49 peptidase for the hydrolysis of Arg_2-2NA by two orders of magnitude (207-fold in Y327A, 138-fold in Y327F) relative to that of the wild-type enzyme (Table 2). The K_m value increased 2-fold in Y327A, and no significant change in K_m was observed when Tyr^{327} was substituted by Phe. A very similar result was obtained previously for the human counterpart: Y318F variant showed a 100-fold lower k_{cat} value compared to wild-type, and no change in K_m value.[9] Interestingly, the Y318A variant of the human enzyme could not be expressed in *E. coli*, whereas the yeast Y327A variant was readily available. The reduction of catalytic efficiency (k_{cat}/K_m) was stronger when Tyr^{327} was replaced by Ala (460-fold *versus* 175-fold). The increase of K_m, observed with Y327A, indicated the importance of Tyr^{327} for ligand binding.

The function of a conserved tyrosine outside the zinc-binding motif was investigated in metallopeptidases of several other families. It was found to be essential for catalytic activity of aminopeptidase A and leukotriene A_4 hydrolase,[17,18] both members of family M1, of neurolysin and thimet oligopeptidase (family M3).[19] Furthermore, Tyr at position 157 is proposed to stabilize the transition state in thermolysin (family M4) as deduced from the crystallographic structures of enzyme-inhibitor complexes.[20]

Affinity for Valorphin

Heptapeptide valorphin, H-Val-Val-Tyr-Pro-Trp-Thr-Gln-OH, is a naturally occurring, endogenous opioide peptide of the hemorphin family. Hemorphins are derived from the β-globin chain of hemoglobin.[21] Valorphin is among the peptides for which human metallopeptidase M49 (dipeptidyl peptidase III, DPP III) showed the highest affinity (K_i ~ 50 nmol dm^{-3}).[22] At the same time, valorphin was a very poor substrate of the human enzyme.

To investigate further the potential role of Tyr^{327} and Glu^{461} in ligand binding, the affinity of the yeast wild-type and enzyme variants for this heptapeptide was determined by measuring the hydrolysis of Arg_2-2NA and treating valorphin as alternate substrate inhibitor.[14] The determined K_i value of the yeast wild-type enzyme was 6.8-fold higher than that of its human counterpart.[22] The affinity of the E461Q mutant was decreased 25-fold, and of the Y327A and Y327F, 17-fold and 15-fold, respectively compared to wild-type (Table 4), indicating the involvement of both Glu^{461} and Tyr^{327} in peptide binding to yeast DPP III.

In contrast to Glu^{461}, which is 3.9 Å apart from the catalytic zinc ion, Tyr^{327} is not situated in the vicinity of the catalytic site in ligand-free yeast peptidase M49 (the distance to Zn^{2+} is 21.4 Å) (Figure 3). Recently, the

Table 4. Affinity of wild-type yeast M49 peptidase and its mutants for valorphin

Enzyme form	K_i / μmol dm^{-3}
Wild-type	0.34 ± 0.03
E461Q	8.36 ± 1.52
Y327A	5.85 ± 0.21
Y327F	5.10 ± 0.27

K_i values were determined with Arg-Arg-2NA as substrate at pH = 8.0, according to Chu and Orlowski.[14]

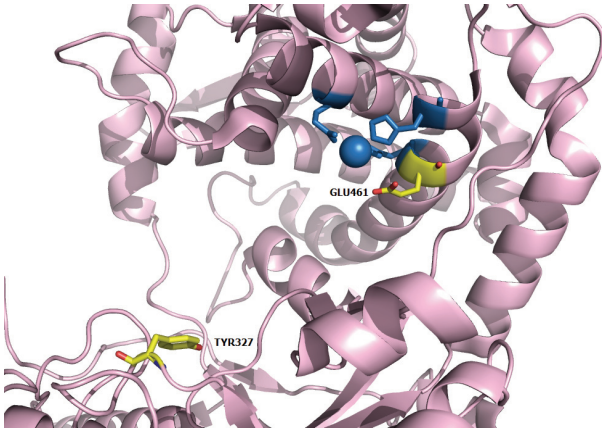

Figure 3. Detail of the ligand-free yeast peptidase M49 crystal structure showing the catalytic zinc ion (blue sphere), conserved Glu[461] and Tyr[327].

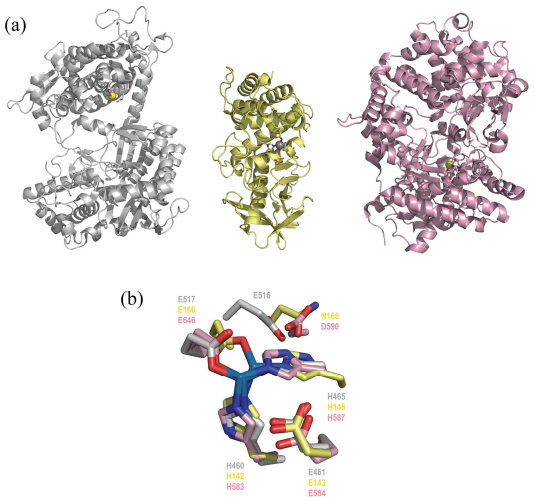

Figure 4. Crystal structures of the yeast M49 peptidase (PDB code: 3csk), grey; thermolysin (PDB: 1 tmn), yellow, and neprilysin (PDB code: 1 dmt), pink (a). Superposition of the zinc–coordinating residues of DPP III, thermolysin (PDB code: 1 tmn) and neprilysin (PDB: 1 dmt) (b).

crystallographic structure of the human M49 peptidase-ligand complex revealed that binding of the pentapeptide tynorphin (Val-Val-Tyr-Pro-Trp) induces a large domain motion in human enzyme.[8] The conserved Tyr[318], being 21.3 Å apart from the zinc-ligating His[450] residue in the free enzyme, thereby approaches to the distance of 6.5 Å, and contributes in ligand binding. Since the yeast and human peptidase M49 share the same protein fold, and are highly similar in the active-site region, we suppose that the same domain motion occurs in the yeast enzyme, which rationalizes the functional importance of Tyr[327].

The contribution of the glutamic acid residue of the active-site motif HEXXGH to the ligand binding of human and yeast M49 peptidase was indicated by our recent MD simulations.[6,7] Our present study, revealing much weaker binding of valorphin to the mutant E461Q compared to the wild-type yeast enzyme, represents the first experimental evidence for the role of this Glu in substrate binding, since the only crystal structure of the enzyme-ligand complex was obtained with the E451A variant.

Due to the dramatic decrease (9200-fold) observed in E461Q k_{cat}, Glu[461] is a residue essential for catalytic activity of yeast M49 peptidase. It is located only 3.9 Å apart of the active-site zinc ion and hydrogen-bonded to the water molecule that is the fourth ligand of zinc in the crystal structure of the yeast enzyme.[5] Structurally equivalent to Glu[461], glutamic acid residue is present in the active sites of two zinc endopeptidases, Glu[143] in bacterial enzyme thermolysin, and Glu[584] in mammalian peptidase neprilysin.[5] For thermolysin, it has been shown that Glu[143] acts as a general base in the deprotonation of the water molecule that attacks the scissile peptide bond.[23] A similar activating role has been ascribed to Glu[584] in neprilysin. Although structurally unrelated (Figure 4a), these three zinc peptidases have a very similar zinc coordination geometry through two histidines and one glutamic acid residue (His[460], His[465] and Glu[517] in the yeast M49 peptidase) (Figure 4b).

CONCLUSION

The role of the two absolutely conserved amino acid residues in the M49 family of proteases was investigated by mutational analysis of the yeast *Saccharomyces cerevisiae* ortholog. Glu[461], situated in the upper protein domain, in the active-site motif HEXXGH, 3.9 Å from the catalytic zinc ion, and Tyr[327], located 21.4 Å from the catalytic center, in the lower protein domain.

The dramatic reduction of the substrate hydroylsis rate (9200-fold decreased k_{cat} for Arg_2-2NA) was observed for the single mutant where Glu[461] has been replaced by a glutamine suggesting that this glutamic acid residue is essential for enzyme catalysis.

The substitution of Tyr[327] by Ala or Phe decreased the k_{cat} of the yeast M49 peptidase by 2 orders of magnitude. The reduction of catalytic efficiency (k_{cat}/K_m) was stronger when Tyr[327] was replaced by Ala (460-fold *vs.* 175-fold).

Affinity of the wild-type and enzyme variants was determined for valorphin, a naturally occurring endogenous opioid peptide (Val-Val-Tyr-Pro-Trp-Thr-Gln) by treating it as alternate substrate inhibitor. The affinity of the E461Q mutant for valorphin was decreased 25-fold, and of the Y327A and Y327F, 17-fold and 15-fold, respectively compared to the wild-type (K_i = 0.34 μmol dm^{-3}), indicating the involvement of both Glu[461] and Tyr[327] in peptide binding to the yeast M49 peptidase.

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