

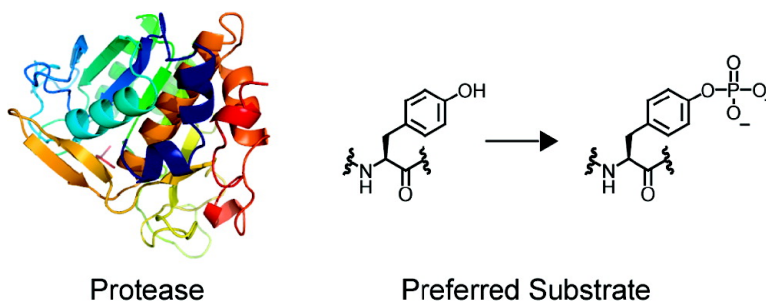
Communication

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## A Remodelled Protease That Cleaves Phosphotyrosine Substrates

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Proteases are the largest family of enzymes in the human genome and play central roles in a wide range of physiological processes.<sup>1</sup> To carry out these diverse functions, proteolytic enzymes have evolved highly tuned substrate specificities that allow them to degrade specific cellular proteins in response to appropriate signals. For many proteases, this specificity is encoded by the amino acid sequence of the polypeptide substrate surrounding the bond that is cleaved (the scissile bond). For example, trypsin recognizes basic residues such as lysine or arginine, and a great diversity of such substrate preferences has been described.<sup>2,3</sup> Remarkably, the substrate specificity of known proteases is limited to recognition of the 20 proteogenic (unmodified) amino acids: no protease has been shown to preferentially cleave its substrates adjacent to a site of post-translational modification, such as phosphorylation. Such enzymes would potentially find widespread application either as analytical tools for proteomics<sup>4</sup> or as catalysts for the synthesis of novel biopolymers.<sup>5–7</sup>

Tyrosine phosphorylation is a dominant signaling mechanism in metazoans that links extracellular hormonal cues to the activation of intracellular signaling pathways. Herein we report efforts to design a protease that selectively cleaves its substrates adjacent to phosphotyrosine residues. Site-directed mutagenesis has been widely used to probe and modify the catalytic properties of enzymes,<sup>8–13</sup> and the bacterial protease subtilisin BPN' was a model system for many early efforts in protein design.<sup>12–19</sup> Subtilisin variants have been identified that display enhanced activity in organic solvents,<sup>20,21</sup> stability at extremes of pH<sup>14,21</sup> or temperature,<sup>15,21</sup> and resistance to chemical oxidation.<sup>16</sup> Subtilisin's catalytic properties have been altered to introduce peptide ligase<sup>5,6</sup> or peroxidase<sup>22,23</sup> activity, and its substrate specificity has been tuned to favor specific hydrophobic or electrostatic features.<sup>17–19</sup> Many of these changes have been rationalized through structural analysis of subtilisin mutants, making this enzyme a well-characterized template for the design of novel proteolytic specificity.

Wild-type subtilisin BPN' exhibits a strong preference for peptide substrates containing phenylalanine or tyrosine at the residue directly N-terminal to the scissile bond (the P1 position). This specificity is controlled by residues that line a substrate binding pocket in the protease (the S1 pocket) that makes contact with the P1 aromatic side chain. We sought to remodel the S1 substrate binding pocket of subtilisin BPN' to selectively enhance its activity toward phosphotyrosine substrates.

A panel of peptide substrates were synthesized in which the P1 residue was varied as either phosphotyrosine, tyrosine, or phenylalanine. The residues surrounding P1 were fixed to an optimal subtilisin substrate sequence in order to direct peptide cleavage N-terminal to the P1 residue.<sup>24</sup> Finally, the aminobenzoic acid (Abz)–nitrotyrosine (Y<sup>NO<sub>2</sub></sup>) FRET donor–quencher pair was

incorporated so that peptide cleavage could be monitored by fluorescence.<sup>24</sup> Using these substrates, wild-type subtilisin BPN' cleaved a phosphotyrosine peptide approximately 500-fold less efficiently ( $k_{\text{cat}}/K_{\text{M}}$ ) than the matched tyrosine or phenylalanine substrates (Figure 1).

Modeling of a phosphotyrosine side chain into the P1 pocket of subtilisin suggested multiple residues that could potentially make contacts with the tyrosine phosphate. We therefore systematically mutated the residues lining the S1 pocket of subtilisin to introduce basic residues or hydrogen bond donors that might make favorable interactions with the negatively charged phosphotyrosine moiety. These 18 single mutants were expressed, purified, and assayed against model peptide substrates.

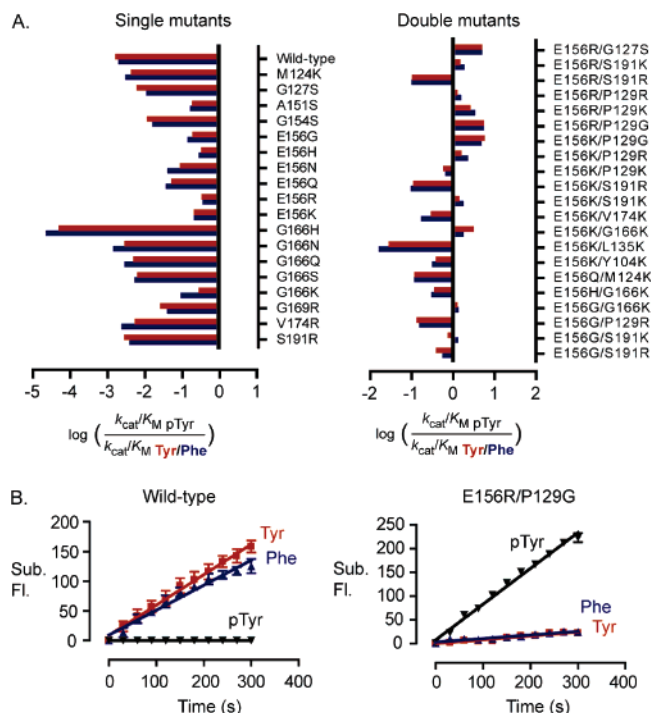
Mutations at several positions in the S1 pocket enhanced selectivity for phosphotyrosine substrates. Mutation of Glu156 to the basic residues arginine or lysine had a dramatic effect on substrate selectivity, resulting in ~200-fold enhancement in selectivity for phosphotyrosine relative to tyrosine or phenylalanine (Figure 1). This improvement was achieved primarily through a reduction in the  $K_{\text{M}}$  for phosphotyrosine peptides (Supporting Information Table 1). Glu156 sits in a solvent-exposed region at the entrance to the S1 binding cleft, and modeling suggests that the side chain of Glu156 would be in close proximity to the phosphate moiety of bound phosphotyrosine substrates (Supporting Information Figure 1).

To search for variants with further selectivity for phosphotyrosine, Glu156 mutations were combined with second site mutations to generate a panel of 21 double mutants that were expressed, purified, and assayed (Figure 1). Several of these double mutants showed greater than 10-fold additional enhancements in phosphotyrosine selectivity, resulting in enzymes that preferentially cleave phosphotyrosine relative to the optimal wild-type substrate. The most selective mutants combine an E156R or E156K mutation with the substitution P129G. Pro129 sits at the entrance to the S1 binding cleft directly opposite Glu156, where the cavity created by the P129G mutation could potentially accommodate the phosphate moiety (Supporting Information Figure 1). The double mutant E156R/P129G cleaved a phosphotyrosine substrate 5-fold more efficiently than the optimal wild-type substrate, representing a greater than 2500-fold switch in substrate selectivity relative to the wild-type enzyme. The double mutant enzyme is expressed at levels comparable to the wild-type and has high specific activity ( $k_{\text{cat}} = 37.1 \text{ s}^{-1}$ ), suggesting that the selectivity improvement for phosphotyrosine is achieved without impairing the stability or catalytic efficiency of the enzyme.

We next sought to characterize how these mutations influence the selectivity of the double mutant for alternative P1 substrates. Optimal peptide substrates were synthesized containing sulfotyrosine (sTyr), phosphoserine (pSer), and phosphothreonine (pThr) amino acids at the P1 position, and the E156R/P129G enzyme was

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**Figure 1.** (A) Plot of the log ratio of catalytic efficiencies for subtilisin single and double mutants assayed with the peptide substrates Abz-FRPXGFY<sup>NO2</sup>-D, where X is either Tyr, Phe, or pTyr. (B) Cleavage of the Abz-FRPXGFY<sup>NO2</sup>-D peptide (0.8  $\mu$ M) as a function of time by wild-type and E156R/P129G subtilisin. Y axis: substrate fluorescence.

**Table 1.** Catalytic Efficiency of E156R/P129G Subtilisin with Peptide Substrates

Abz-FRPXGFY <sup>NO2</sup> -D		Suc-AAPX-pNA	
P1	$k_{cat}/K_M$ ( $M^{-1} s^{-1}$ )	P1	$k_{cat}/K_M$ ( $M^{-1} s^{-1}$ )
pTyr	$1.41 \times 10^7$	Phe	$8.64 \times 10^4$
sTyr	$7.27 \times 10^6$	Leu	$2.26 \times 10^3$
Tyr	$2.71 \times 10^6$	Ala	$5.93 \times 10^2$
Phe	$2.71 \times 10^6$	Val	$1.89 \times 10^1$
pSer	$3.90 \times 10^2$	Ile	$2.33 \times 10^1$
pThr	$1.85 \times 10^1$	Asp	$1.29 \times 10^2$
		Glu	$3.53 \times 10^2$
		Arg	$1.30 \times 10^1$
		Lys	$5.28 \times 10^1$

assayed against these substrates (Table 1). Sulfotyrosine was utilized only 2-fold less efficiently than phosphotyrosine, consistent with the fact that these amino acids are isosteric and negatively charged at neutral pH. By contrast, phosphothreonine and phosphoserine peptides were extremely poor substrates for the modified enzyme, with catalytic efficiencies diminished approximately 36 000-fold and 76 000-fold, respectively, relative to the matched phosphotyrosine peptide. The narrow S1 binding cleft of subtilisin makes close hydrophobic interactions with the aromatic P1 side chain of the substrate that sterically preclude the binding of  $\beta$ -branched amino acids at this site; the selectivity we observe relative to phosphoserine and phosphothreonine suggests that these interactions have been preserved in the double mutant.

We further characterized the selectivity of E156R/P129G by assaying its activity against a commercial panel of protease

substrates that vary in the identity of the P1 amino acid (Table 1). Within this panel, phenylalanine was the optimal residue at P1, suggesting that the selectivity of E156R/P129G for non-phosphorylated substrates was not significantly altered relative to the wild-type enzyme. Importantly, aspartate and glutamate remained very poor P1 substrates for the modified enzyme (utilized 670 and 245-fold less efficiently relative to phenylalanine), indicating that the mutations which enhance phosphotyrosine binding do not nonspecifically bias the enzyme toward all acidic substrates.

The identity of the P1 residue is a critical determinant of substrate binding to subtilisin BPN'. However, amino acids at adjacent positions contribute to substrate recognition,<sup>24</sup> and it will be important to explore how these preferences affect the cleavage patterns produced when complex mixtures of proteins are used as substrates. Alternatively, as the selectivity of adjacent subsites has been modified through protein engineering,<sup>25,26</sup> it may be possible to introduce additional mutations that confer recognition of specific phosphotyrosine consensus sequences.

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**Supporting Information Available:** Supplementary Table 1 and Figure 1 as well as complete experimental protocols. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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