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Engineering protease specificity made simple, but not simpler

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Abstract

Engineering protease specificity has been a long sought research goal. New findings on OmpT, an outer membrane protease from *Escherichia coli*, reveal the remarkable success of a simple strategy.

Genes encoding for proteolytic enzymes make up 2–4% of the typical genome¹. This formidable arsenal comes with exquisite substrate specificity for a limited set of amino acid residues. Engineering such specificity in proteases for reagent, diagnostic and therapeutic gains has been a long sought research goal. Successes have been rare, and only a limited consensus has emerged on how protease specificity is encoded in protein structure, or how it can be redesigned rationally. In this issue of *Nature Chemical Biology*, Varadarajan *et al.*² report the breakthrough observation that selective preference for the P1 residue³ of a substrate can be engineered with minimal amino acid substitutions within the S1 pocket of OmpT, an outer membrane protease from *E. coli*. The conclusion is refreshingly simple and carries far-reaching consequences for protein engineering of other enzyme systems.

OmpT belongs to a growing family of proteases implicated in the pathogenicity of Gram-negative bacteria, such as *E. coli*, *Salmonella typhimurium* and *Yersinia pestis*⁴. Initially regarded as a nuisance for the purification of recombinant proteins expressed in *E. coli*, OmpT garnered attention for its ability to cleave substrates at Arg-Arg bonds, similar to proprotein processing peptidases such as kexin and furin⁵. OmpT is shaped like a vase. It is built as a 70-Å-long, 10-stranded antiparallel β-barrel long enough to span the outer membrane of *E. coli* and to present its catalytic domain to solvent⁶. Two pairs of residues sit on the opposite sides of the hollow barrel and define the active site groove (Fig. 1). The His212-Asp210 pair resembles the arrangement found in the serine protease His-Asp-Ser triad, and the Asp83-Asp85 pair resembles the active site of an aspartic protease. The Asp83-His212 dyad activates a water molecule for nucleophilic attack on the Arg-Arg peptide bond. The S1 site, which is specific for arginine and lysine residues, is shaped as a deep pocket containing Glu27 and Asp208 at its bottom and Ser223 nearby. The S1' site is less well defined, underscoring the rather broad preference for arginine but also lysine, isoleucine and histidine at this position.

Varadarajan *et al.*² attacked the problem of redesigning the P1 specificity of OmpT using a brute-force approach that exploits saturation mutagenesis and error-prone PCR to alter the chemistry of the S1 pocket. The goal was an ambitious one: convert the P1 specificity of OmpT from arginine to glutamate (complete charge reversal), tyrosine (hydrophobe) or threonine (small polar)—three residues toward which the wild type shows no catalytic activity. Large libraries of mutants were screened using a clever strategy that identifies the desired substrate activity against a reference counterselection substrate carrying wild-type preference⁷. The entire approach is conceptually simple, but technically no simpler than alternative strategies used for other enzyme systems⁸. Remarkably, the authors succeeded in all three cases and

identified mutants of OmpT that cleave at the Glu-Arg (ER-OmpT), Tyr-Arg (YR-OmpT) and Thr-Arg (TR2-OmpT) bonds with values of $k_{\text{cat}}/K_{\text{m}}$ that are at least as good as that found for cleavage of Arg-Arg by wild type ($2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$). The Glu-Arg preference of ER-OmpT deserves special mention because it has no counterpart in any natural protease. Importantly, the gain in P1 specificity comes with exquisite selectivity, as each mutant shows negligible or no activity toward the Arg-Arg bond preferred by wild type. For the first time, the P1 specificity of an enzyme has been redesigned without loss of catalytic activity or the exquisite selectivity found in natural proteases.

It is quite instructive to dissect how this impressive result was achieved at the molecular level. ER-OmpT carries the triple substitution E27L D208R S223G, which reverses the polarity of the S1 site with a +3 net charge substitution (Fig. 1). The negative charges of Glu27 and Asp280 at the bottom of the S1 pocket are replaced by a positively charged arginine with minimal changes in packing, which apparently ensures engagement of glutamate at the P1 position of the substrate. YR-OmpT carries the substitutions E27W and D208H in the S1 site, and five additional replacements: V29P, I170V, Y172V, Y221A and L265V. The E27W and D208H substitutions make the S1 site more hydrophobic, but the changes in Tyr221, Leu265 and Val29 also widen access to the S1 pocket without compromising its gain in hydrophobicity. Optimal engagement of tyrosine at P1 requires further repacking of the S1 pocket with replacement of Tyr172 beneath residue 27. An additional round of error-prone PCR generated TR2-OmpT, which carries the substitutions E27H, D208L and S223D in the S1 site and the six additional replacements V29S, D214N, P243S, W253G, N270Y and S276G. In this case the changes within the S1 site and its entrance relocate a negative charge and maintain an overall polarity and packing for favorable interaction with threonine at the P1 position. The recipe for redesigning the P1 specificity of OmpT is simple, a posteriori: change the polarity of the S1 pocket and repack it by acting directly on the residues that define the S1 site (Glu27, Asp280 and Ser223) and its entrance (Val29, Tyr221 and Leu265). It would be of great interest to establish whether saturation mutagenesis at all these six residues is necessary and sufficient to generate the entire gamut of P1 specificities accessible to natural proteases.

But the real test for this new paradigm will come from other enzyme scaffolds. An intriguing connection exists between OmpT and serine proteases in the architecture of the S1 pocket and its position relative to the catalytic machinery. Within the same fold, trypsin-like proteases prefer arginine and lysine side chains at the P1 position, but chymotrypsins prefer tyrosine and tryptophan residues⁵. At the bottom of the S1 cavity, trypsins carry Asp189 and chymotrypsins carry Ser189. Earlier studies have shown that conversion of P1 specificity between trypsin and chymotrypsin requires the expected D189S replacement, but also extensive additional replacements at residues that do not shape the S1 pocket⁹. The same strategy, however, fails in the reverse conversion of chymotrypsin into trypsin¹⁰. Recent mutagenesis studies have shown that the simple S189D replacement in chymotrypsin is sufficient to confer trypsin-like specificity when access to the S1 pocket is enhanced with the A226G substitution¹¹—a result that echoes the remarkable findings by Varadarajan *et al.*² on OmpT. The S189D A226G mutant of chymotrypsin is a poor protease (it is 5,000-fold slower than trypsin at cleaving substrates with lysine at P1), but it is a step in the right direction. In the end, creating the desired protease specificity may require a clever screen. That should make rationally designing protease specificity simple, but not simpler.

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Figure 1.

Surface representation of OmpT viewed from above the plane of the membrane, down the β -barrel axis. The structure is taken from Protein Data Bank accession code 1178 (ref. 6). The active site is shaped as a wide aperture with two pairs of catalytic residues (Asp210-His212 and Asp83-Asp85, in gold) facing each other from the opposite sides of the barrel. The acidic S1 pocket (residues in red) comprises Glu27, Asp208 and Ser223 at the bottom of a cavity accessible from a rim lined up by the hydrophobic residues (in orange) Val29, Tyr221 and Leu265 and accounts for the arginine preference at the P1 position of the substrate. Mutagenesis of these six residues converts the P1 specificity of OmpT from arginine to glutamate, tyrosine or threonine². Additional residues mutated in some constructs are located away from the S1 pocket and are shown in cyan (some residues are not visible in the orientation shown). The P1 specificity of OmpT can be engineered by changing the polarity and repacking of the S1 pocket. Varadarajan *et al.*² also constructed OmpT mutants with new P1-P1' specificity. Notably, RV-OmpT is active toward Arg-Val peptide bonds present in the antimicrobial peptide human β -defensin3 and can cleave the Arg561-Val562 bond in plasminogen at a rate comparable to that of the natural enzyme tissue-type plasminogen activator.