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Proteases That Can Distinguish among Different Post-translational Forms of Tyrosine Engineered Using Multicolor Flow Cytometry

Navin Varadarajan,[†,⊥] Mark Pogson,[‡] George Georgiou,*[,†,‡,§] and Brent L. Iverson*[,‡,⊥]

Department of Chemical Engineering, Institute for Cellular and Molecular Biology, Department of Biomedical Engineering, and Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas 78712

Received September 25, 2009; E-mail: gg@che.utexas.edu; biverson@mail.utexas.edu

Abstract: We report the isolation of a highly active ($k_{cat}/K_M \sim 10^5$ $M^{-1}s^{-1}$) variant of the *E. coli* endopeptidase OmpT that selectively hydrolyzes peptides after 3-nitrotyrosine while effectively discriminating against similar peptides containing unmodified tyrosine (160-fold), sulfotyrosine (3600-fold), phosphotyrosine (>8000-fold), and phosphoserine (>8000-fold). The isolation of endopeptidase variants that can discriminate between substrates based on the post-translational modification of Tyr was made possible by implementing a multicolor flow cytometric assay for the screening of large mutant libraries. For the multicolor assay, a desired selection substrate was used simultaneously with multiple counterselection fluorescent substrates to isolate rare enzyme variants that displayed finely tuned substrate specificity. This work demonstrates that enzymes with exquisite selectivity can be isolated from large libraries using appropriate high throughput screening approaches and constitutes a critical step toward the production of a 3-nitrotyrosine-specific protease useful for proteomic applications.

Introduction

Proteases constitute the largest enzyme family with ~2000 identified members. In humans alone there are ~560 proteases, accounting for ~2% of the genome.[1] Proteases play fundamental roles in almost every cell function including migration, adhesion, apoptosis/degradation, and signaling.[2] Therefore, it is not surprising that proteases exhibit a wide range of substrate preferences, catalytic activities, and selectivities.[1,2] Unfortunately, however, the routine application of proteases in a wide variety of contexts from proteomics and biotechnology to therapeutics is restricted to only a small fraction of proteases such as the subtilisins, trypsins, and carboxypeptidases. Engineering programmed specificity into stable protease scaffolds holds promise as a route to a new generation of useful proteases. For example, properly engineered proteases can be envisioned as the protein-cleaving equivalents of restriction endonucleases, with the potential to revolutionize proteomic research.[3] As such, selectivity is a major consideration, because secondary/off-target cleavages will preclude any widespread application. General high-throughput library screening assays are the first step toward isolating highly active protease variants possessing the desired new activity, but for practical applications it is likely that a counterselection strategy will be required to engineer proteases with the appropriate selectivity.[4−6]

The utility of counter-selection in programming enzyme selectivity is now widely recognized, but its implementation in screening experiments remains challenging from a technical standpoint.[7] Consequently, there have been few screening experiments that profile enzymic reactivity across more than one substrate, and those that have been reported have been primarily restricted to dual selections, i.e. they involve a single selection and a single counterselection substrate.[8,9] Although such dual screening experiments might prove sufficient for certain enzymes, protease engineering will necessitate the use of multiple counterselections to engineer precise selectivity.[3,10] A multiple simultaneous counterselection approach has at least two significant advantages compared to a tiered strategy using separate rounds with different individual counterselection substrates: (1) The simultaneous multicounterselection approach is potentially far less time and resource intensive compared to a tiered approach and (2) the most selective overall clone may not be the most active in any single counterselection experiment,

[†] Department of Chemical Engineering.
[‡] Institute for Cellular and Molecular Biology.
[§] Department of Biomedical Engineering.
[⊥] Department of Chemistry and Biochemistry.

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increasing the risk it will be lost in an early round by being outcompeted due to an excess of more active clones with reduced selectivity.

Current proteomic methodologies for detecting post-translational modifications are based mostly on tandem mass spectrometry (MS/MS) to sequence individual peptides generated via proteolysis using standard proteases such as chymotrypsins, trypsins, and carboxypeptidases. However, since post-translational modifications can be labile, it is still often difficult to identify all modification sites[11,12] during a proteomic analysis. One potential work-around to this problem would be a set of proteases that only cleave at a site of modification, followed by either mass spectrometry or even fingerprint analysis using N-terminal sequencing to identify the site of cleavage and therefore the site of modification. Unfortunately, no naturally occurring, post-translational modification-specific proteases have been reported.

We and others have been pursuing an enzyme-engineering-based approach to produce the first proteases specific for substrates with post-translational modifications.[10,13] For example, Shokat and co-workers were able to engineer a bacterial protease subtilisin BPN′ variant with enhanced activity for phosphotyrosine (pTyr)-containing substrates.[13] We have previously reported an *E. coli* outer membrane protease (OmpT) variant capable of recognizing sulfotyrosine (sTyr). Although the sTyr-specific variant displayed remarkable selectivity over pTyr (200-fold), its selectivity in comparison to unmodified tyrosine was only modest (10-fold).[10]

Posttranslational oxidation of tyrosine to 3-nitrotyrosine (nTyr), mediated by reactive nitrogen species, has been recognized as a biomarker for neurodegenerative and chronic inflammatory diseases such as Parkinson's disease, Alzheimer's disease, cancer, and ocular inflammation.[14] Nitration of tyrosine causes a dramatic shift in the (side chain) pK_a from ~10 to ~7 and is usually associated with a decrease or loss in protein function.[15] Additionally, nitration is believed to modify the propensity of tyrosine to be phosphorylated thus permanently altering its signaling capability.[16] Unlike phosphorylation/sulfation that are catalyzed by enzymes with preferred recognition sequences, nitration of tyrosine and other amino acids such as cysteine is the result of reactive nitrogen species that originate from NO,[14] and only certain Tyr residues appear to be susceptible to nitration. Currently, identifying nitration sites in proteins is not trivial.[14] The lack of convenient tools that can identify nTyr containing peptides/proteins has been an obstacle in understanding its potential mechanistic contribution to inflammation/disease.[16]

Herein, we report the engineering of enzymes that selectively hyrdolyze peptides with a nTyr residue in P1 with rates (k_{cat}/K_M ~10^5 M^{-1} s^{-1}) that are well over 2 orders of magnitude higher than peptides containing pTyr, sTyr or unmodified Tyr. Key to the isolation of such enzymes was the implementation of a multicolor flow cytometric assay that enabled the selection of the desired activity with simultaneous counterselection against two (or potentially more) substrates. This work represents the first critical step toward the production of a nTyr-specific protease useful for proteomic applications.

Results

The nTyrR selection substrate, **1a**, contained a cleavage site (nTyr↓Arg) flanked by a fluorophore (Atto633, λ_{em} = 657 nm) and a positively charged tail on one side, and a dark quencher (QSY21) on the other. Cleavage after nTyr by any of the surface-displayed OmpT variants resulted in the capture of the positively charged, red-fluorescent portion of the substrate, thereby labeling the cells expressing an active variant red-fluorescent (Figure 1). Using the same scheme but different fluorescent dyes, three different counter-selection substrates (CSub) containing Tyr↓Arg (**2a**)/sTyr↓Arg (**3a**)/pTyr↓Arg (**4a**), were synthesized and flanked by BODIPY (λ_{em} = 505 nm) and QSY7 (Figure 1B). Cleavage at any of the CSubs lead to the electrostatic capture of an unquenched BODIPY-containing product, rendering the cells green fluorescent. Another CSub using a zwitterionic, fluorescently labeled (tetramethyl rhodamine, orange fluorescent) peptide containing the dibasic Arg↓Arg sequence (**5a**) preferred by the wild-type (WT) OmpT was used to monitor parental-like activity. Simultaneous incubation of cells expressing OmpT variants with multiple substrates labeled the cells according to the selectivity profile of the surface-expressed variants, with red-only fluorescence indicating the desired selective variant, and any other combination indicating reduced selectivity and therefore undesired variants. The ability of the assay to report enzymic activity and selectivity accurately was confirmed using the previously reported OmpT-YR variant[3] (specific for cleavage between tyrosine and arginine residues) simultaneously incubated with 20 nM Glu↓Arg-BQ7 (**7a**), 20 nM Tyr↓Arg-AtQ21 (**6a**), and 100 nM Arg↓Arg-TMR (**5a**) (Supporting Information Figure S1).

A partial saturation library targeting 21 amino acids encompassing the entire OmpT active-site[17] was constructed using oligonucleotide-based gene synthesis by mixing oligonucleotides containing the degenerate NNS codon (N = A, T, G, C; S = G, C) with wild-type oligonucleotides at a 9:1 molar ratio, respectively. The resulting library was transformed into electrocompetent *E. coli* BL21(DE3), an *ompP ompT* deficient strain, to yield ~10^9 transformants (3-fold library coverage).[10] The cells were grown at 37 °C to an OD_{600} of 2 units, and a 1 mL aliquot of the culture in 1% sucrose was incubated with 20 nM **1a**, 20 nM **2a**, and 100 nM **5a**. Gates were set based on forward/side scatter, red (Atto633, 660/20 nm), green (BODIPY, 530/30 nm), and orange (TMR, 575/25 nm) fluorescence channels to isolate *E. coli* cells labeled exclusively red, isolating ~1% of the population. Subsequent rounds of sorting were performed using 20 nM **1a**, 20 nM **2a** (rounds 2 and 5)/20 nM **3a** (round 3)/20nM **4a** (round 4), and 100 nM **5a** (Figure 2, for details of gating and sorting strategy please refer to Supporting Information Figures S2 and S3). After five rounds of regrowth and sorting, the isolated cells were plated. Subsequent flow-cytometric analysis of single clones (Figure 3) identified a highly active and selective OmpT variant designated OmpT-nYR. DNA sequencing revealed OmpT-nYR contained a total of seven amino acid changes including both an acidic and aromatic amino acid in the putative S1 binding pocket (Table 1).

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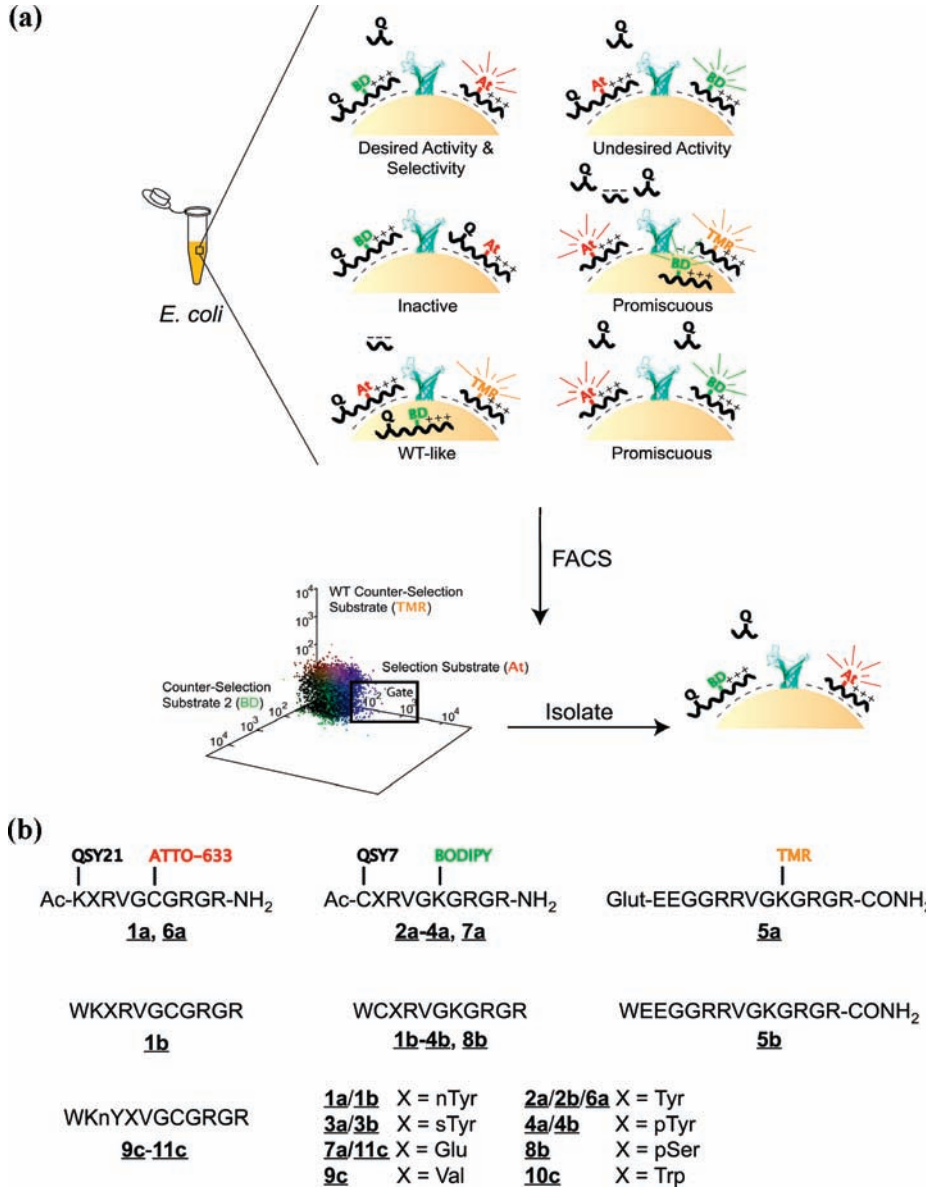

Figure 1. (A) Three-color flow-cytometric assay to engineer selectivity across multiple substrates. BD = BODIPY, TMR = tetramethylrhodamine, At = Atto 633, Q = Dark quencher. (B) Sequences of the substrates used for screening and characterization.

The OmpT-nYR variant was purified (>90% purity as judged by SDS-PAGE) by extraction with β-octyl glucoside and the kinetics of hydrolysis of nonfluorescently labeled peptide substrates **1b**−**5b** and **8b** were determined. Consistent with its fluorescence profile and the sorting criteria, OmpT-nYR demonstrated efficient hydrolysis of nTyr↓Arg (**1b**, k_{cat}/K_M 8 ± 5 × 10^4 M^{-1} s^{-1}) as well as high-selectivity based on greatly diminished cleavage activity with the substrates containing Tyr↓Arg (**2b**, 160-fold), sTyr↓Arg (**3b**, 3600-fold), pTyr↓Arg (**4b**, >8000-fold), and Arg↓Arg (**5b**, >8000-fold) (Table 1). OmpT-nYR also showed no cross-reactivity with the phosphoserine peptide, **8b**. Two particularly important features of the OmpT-nYR variant are that (1) the overall catalytic efficiency is comparable to WT OmpT with its preferred dibasic substrate **5b** (Table 1) and (2) cross-reactivity was substantially eliminated by the designed concurrent counter-selection criteria.

OmpT-nYR specificity was evaluated at P1′ using substrates **9c**-**11c**. Although, the enzyme displayed some activity against a substrate with Val in P1′ (**9c**), an activity present in wild-type OmpT, no hydrolysis was observed for nTyr↓Trp (**10c**) or nTyr↓Glu (**11c**) (Supporting Information Table S1), indicating the wild-type OmpT preference for a basic residue in P1′ likely remains intact in OmpT-nYR.

Discussion

In order to facilitate the isolation of an engineered OmpT variant that could selectively distinguish nTyr (target post-translational modification) from sTyr, pTyr, Tyr (unmodified), and Arg (WT OmpT preferred) at P1, a multicolor, flow-cytometric assay was designed, capable of reporting activities with multiple substrates simultaneously (Figure 1). A partial saturation mutagenesis library that systematically sampled combinations of different OmpT active site residues was constructed by oligonucleotide-mediated gene-assembly PCR. A 9:1 (randomized:wild-type oligonucleotide) molar ratio (across all 21 active site residues) produced an average of 10 amino acid mutations per gene and allowed efficient exploration of sequence space while still retaining protein folding/function in

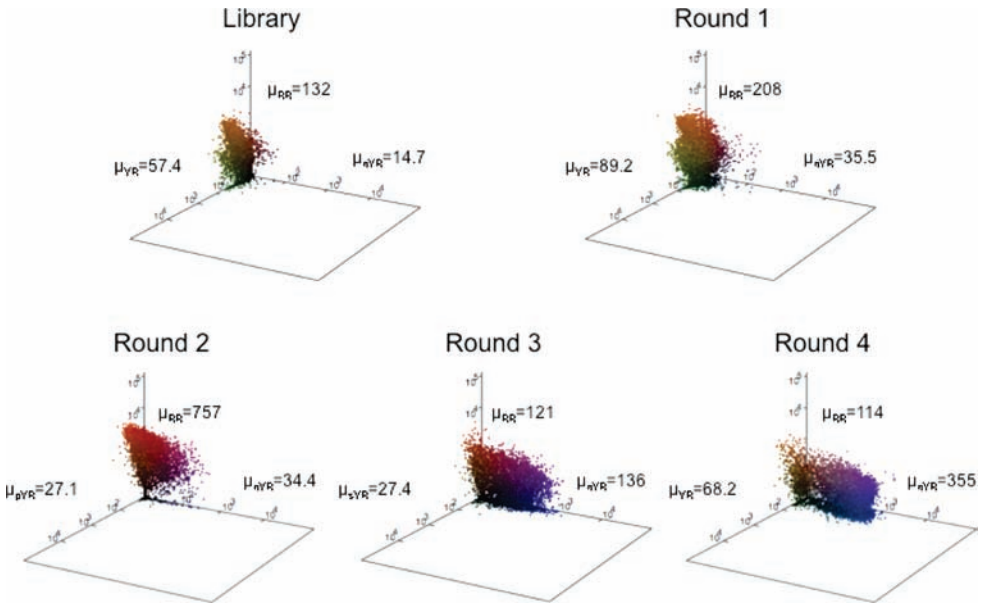

Figure 2. Flow-cytometric data (10 000 events) from the different rounds of sorting, labeled with the indicated substrates.

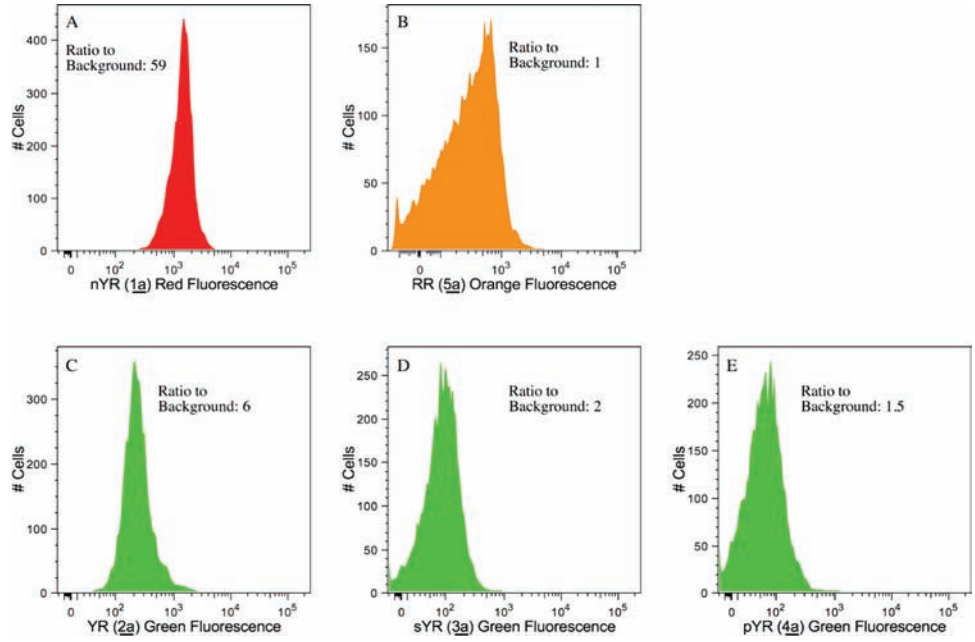

Figure 3. Flow-cytometric data for the OmpT-nYR variant. Fluorescence histograms showing *E. coli* expressing OmpT-nYR labeled with (A) 20 nM nTyr↓Arg-AtQ21 (**1a**), (B) 100 nM Arg↓Arg-TMR (**5a**), (C) 20 nM Tyr↓Arg-BQ7 (**2a**), (D) 20 nM sTyr↓Arg-BQ7 (**3a**), and (E) 20 nM pTyr↓Arg-BQ7 (**4a**).

Table 1. Kinetic Characterization of OmpT-nYR[a]

enzyme	mutations	substrate, cleavage site	k_{cat}/K_M (M^{-1} s^{-1})	selectivity
WT OmpT	–	**5b**, Arg↓Arg	$(1.7 \pm 0.4) \times 10^5$ [b]	
OmpT-nYR	V39G, E153S, I170 V, G196S, D208F, S223G, L265G	**1b**, nTyr↓Arg	$(8 \pm 5) \times 10^4$	
		2b, Tyr↓Arg	$(5 \pm 3) \times 10^2$	160-fold
		3b, sTyr↓Arg	22 ± 6	3600-fold
		4b, pTyr↓Arg	<10	>8000-fold
		5b, Arg↓Arg	no measurable activity	>8000-fold
		8b, pSer↓Arg	no measurable activity	>8000-fold

[a] Amino acid changes and kinetic parameters for WT OmpT and the OmpT-nYR variant, measured at room temperature (25 °C). [b] Data from ref 10.

a significant fraction of library members. The OmpT library was expressed on the surface of *E. coli* and screened using the flow-cytometry assay. High activity for the desired nTyr substrate was achieved on the basis of fluorescence color, thereby excluding reactivity toward sTyr/pTyr/Tyr/Arg. To increase stringency, the combination of counterselection substrates was systematically varied through the different rounds of sorting (Supporting Information Figure S2). After five rounds, cells expressing a highly active and selective variant, OmpT-nYR, were isolated (Figure 3).

The putative S1 pocket of OmpT-nYR contained both an aromatic residue (Asp208Phe), capable of hydrophobic or possibly aromatic

donor−acceptor interactions, and an acidic residue (Glu27). Although the crystal structure of WT OmpT has been previously reported, our efforts to obtain cocrystals of substrate analogues bound to OmpT-nYR variants have been largely unsuccessful, precluding assignment of a definitive molecular recognition motif. Nevertheless, using the WT OmpT structure as a guide, the putative S1 binding pocket of OmpT-nYR can be compared with the mutations seen in the previously reported sulfo-tyrosine variant. Both engineered proteins have a phenylalanine (aromatic residue) in S1 (Glu27Phe for sT4 and Asp208Phe for OmpT-nYR), but while sT4 has basic residues (Ile282His and Asp208Arg) to presumably recognize the negatively charged sulfate, OmpT-nYR has an acidic residue (Glu27) (Supporting Information Table S2).

A detailed kinetic characterization, accomplished using unlabeled peptide substrates confirmed that OmpT-nYR is capable of easily distinguishing between nTyr, sTyr, pTyr, and Tyr in the P1 position. In particular, OmpT-nYR preferred nTyr in P1 by 160-fold over Tyr, 3600-fold over sTyr, and greater than 8000-fold over pTyr (no reaction observed within the limits of detection).

It is of interest to compare the P1 substrate specificity of OmpT-nYR with the specificities of WT OmpT and the previously reported sTyr-specific variant sT4, which was isolated using a single counter-selection (Arg-Arg) substrate. The plots shown in Supporting Information Figure S4 provide a visualization of the overall specificity for each enzyme. OmpT-nYR is considerably more specific than sT4 at P1. Presumably, when selectivity between chemically and structurally similar amino acids is required, for example between post-translationally modified tyrosines, the simultaneous multicounterselction approach enables the isolation of variants displaying the desired degree of discrimination. Importantly, the multicounterselection strategy apparently does not produce specificity at the expense of overall activity because OmpT-nYR exhibits a high level of overall catalytic activity (k_{cat}/K_M).

Kinetic characterization of the P1′ specificity of OmpT-nYR demonstrated that the enzyme displayed low activity against a substrate containing Val in P1′ and virtually no activity toward substrates containing a Trp nor Glu in the same position (Supporting Information Table S1). This result is not surprising in light of the fact that the specificity profile of wild-type OmpT is similar at this position and that our screening assay was focused on engineering P1 specificity. Future engineering efforts, starting with the OmpT-nYR variant reported here, will be directed toward creating enzyme variants displaying broad specificity in the P1′ position in an attempt to generate proteomic tools of practical value. This should be a tractable problem given that previous reports from both our lab[3] and others[18] have demonstrated that plasticity can be engineered into OmpT at the S1′ site.

Materials and Methods

Substrate Synthesis. The peptide substrates were purchased from Genscript (Piscatway, NJ). QSY7 C_5 maleimide, QSY21 carboxylic acid, succinimidyl ester, 5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA, SE), and BODIPY-FL-SE were purchased from Invitrogen (Carlsbad, CA). Atto-633 maleimide was purchased from Atto-TEC GmbH (Sigen, Germany).

The conjugation of fluorophores to the substrates **1a**−**7a** was performed essentially as described before.[3]

Library Construction. The *ompT* gene was assembled by PCR using a set of 48 oligonucleotides designed to include a 5′ *Eco*RI and a 3′ *Hin*dIII site for ligation into pDMLE19. Reaction profiles were: 95 °C, 1 min; 54 cycles: 95 °C, 30 s/45 °C, 30 s/72 °C, 30 s; then 72 °C for 10 min. A 2 μL aliquot of the first PCR reaction was used as template to for a second PCR reaction, amplified using the primers 5′ CCGGGAATTCACCATGCGGGCGAAACT-TCTGGGAATAGTC 3′ and 5′ AACAGCCAAGCTTTTAAAAT-GTGTACTTAAGACCAGCAGT 3′. The PCR product was then gel-purified, digested, and ligated into pDMLE19. A 3 μL aliquot of the desalted ligation reaction was transformed with 40 μL of electrocompetent *E. coli* MC1061 (*araD139* Δ (*ara-leu*) 7696 Δ*lac174 galU galK hsr*$^-$ *hsm*$^+$ *strA*^{R}) cells. Following incubation in 1 mL of SOC media (Difco) at 37 °C for 1 h, the entire transformation mixture was plated on LB plates supplemented with 1% glucose and 200 μg/mL ampicillin. After growth at 37 °C for 8 h, the cells were scraped and resuspended in 2 mL of LB, and 1 mL of the cell suspension was used to inoculate 1 L of fresh media as above. The cells were harvested after 5 h of incubation at 25 °C, and plasmid DNA was isolated and then retransformed into electrocompetent *E. coli* BL21(DE3) (F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)). Finally, for library screening, the transformants were grown to an OD_{600} of 2.0 in 1 L of 2xYT medium (Difco) containing 200 μg/mL of ampicillin at 37 °C.

For the construction of the 90% NNS library, a 9:1 mix of the NNS oligonucleotides and the wild-type codon oligonucleotides corresponding to residues 27, 29, 39, 40, 42, 44, 81, 87, 97, 101, 148, 150, 153, 163, 170, 208, 221, 263, 265, 280, and 282 were used to assemble the gene.[17] All oligonucleotides were purchased from IDT DNA Technologies (Coraville, IA).

Library Screening. The plasmid library was transformed into electrocompetent *E. coli* BL21(DE3) (F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)) and the cells grown to an OD_{600} of 2.0 in 1 L of 2xYT supplemented with 200 μg/mL of ampicillin at 37 °C (6−8 h). An aliquot of the cells adjusted to an OD_{600} of 2.0 (1 mL) was centrifuged at 10 000 rpm for 2 min using a microcentrifuge. The supernatant was discarded, and the cells were resuspended in 1 mL of 1% sucrose. The procedure was repeated once, and a 50 μL aliquot of the cell suspension was added to 948 μL of 1% sucrose and labeled using 1 μL of the appropriate selection substrate (**1a**, final concentration 20 nM), 1 μL of the counterselection substrate (**2a/3a/4a**, final concentration 20 nM), and 1 μL of the wild-type counterselection substrate (**5a**, final concentration 100 nM). An 800 μL aliquot of this labeling reaction was mixed with 200 μL of 1% sucrose and analyzed on a BD Biosciences FACSAria flow cytometer. Library sorting was performed on the FACSAria using gates set based upon FSC/SSC and Red, Green, and Orange Fluorescence channels (Supporting Information Figure S2 and S3). A total of ∼1 × 10^7 cells were screened using nine separate labeling reactions, and ∼1% of the most fluorescent cells were sorted into 3 mL of 2xYT media supplemented with 200 μg/mL ampicillin. The cells were grown to an OD_{600} of 2.0 and resorted, and the process was repeated until the mean fluorescence of the highly fluorescent population remained constant. After five rounds of sorting and resorting, the cells were plated on selective media and individual colonies were analyzed on the flow cytometer.

Enzyme Purification and Characterization. Enzyme purification and kinetic analysis were performed as previously described.[3]

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Supporting Information Available: Figures detailing the sort strategy and Tables profiling OmpT-nYR specificity at the P1′ position. This material is available free of charge via the Internet at http://pubs.acs.org.

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