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Expanding the substrate tolerance of biotin ligase through exploration of enzymes from diverse species

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Escherichia coli biotin ligase (BirA) has been harnessed for numerous biotechnological applications, including protein labeling,^{1–3} purification,^{4,5} and immobilization.⁶ BirA catalyzes the ATP-dependent covalent ligation of biotin to a lysine sidechain of a 15-amino acid recognition sequence called the “acceptor peptide” (AP).⁷ Previously, we showed that BirA could be applied to site-specific protein labeling on the surface of living cells by making use of a ketone isostere of biotin that could be ligated to AP fusion proteins, then derivatized with hydrazide or hydroxylamine probes.⁸ Since that work, we have focused on expanding the small-molecule specificity of BirA to incorporate other useful functional groups, such as azides and alkynes, which can be derivatized with even more chemoselective reactions than the ketone.⁹ Because we found that BirA does not ligate diverse structural analogs of biotin to the AP, in this study, we explored the activities of biotin ligases from nine other organisms. We discovered that yeast biotin ligase accepts an alkyne derivative of biotin, while *Pyrococcus horikoshii* biotin ligase utilizes both alkyne and azide biotin analogs. These new ligation reactions demonstrate the differential substrate specificities of ligases from different species, and open the door to novel protein labeling applications.

Due to our interest in novel methodologies for targeting chemical probes to proteins in the cellular context, and the previous work of our lab in exploiting *E. coli* BirA for this purpose,^{1,8} we synthesized or purchased each of the biotin analogs shown in Figure 1. Desthiobiotin azide (DTB-Az) **2** and propargyl biotin (PB) **3**, whose syntheses are shown in Figure 2, contain unique functional group handles. Azides are naturally absent from cells and can be selectively derivatized with strained alkynes or phosphines under physiological conditions,⁹ while alkynes, also absent from cells, can be selectively derivatized with azides *via* [3+2] cycloaddition in the presence of a copper catalyst.¹⁰ Iminobiotin **4** and diaminobiotin **5** exhibit pH-dependent binding to streptavidin and can be used for protein purification.¹¹ Nitrobenzoxadiazole gamma-amino butyric acid **6** is a fluorophore, and iodouracil and thiouracil valeric acid probes (**7** and **8**) are photoactivatable crosslinkers.¹²

We found that wild-type BirA does not ligate analogs **2–8** to the AP (data not shown). Because precedent exists for differential substrate specificity among homologous enzymes from different species,^{13,14} we decided to clone, express, and evaluate biotin ligases from nine other species. All organisms express one or two biotin ligases,^{15,16} which attach biotin to protein domains involved in the transfer of carboxyl groups. In some organisms, the ligase plays an additional role in repressing transcription at the biotin biosynthetic operon or other biotin-sensitive genes.¹⁷ To select our panel of novel biotin ligases, we first noted that biochemical and/or structural data were available for the human,¹⁸ *S. cerevisiae* (yeast),¹⁹ *P. horikoshii*,¹⁶ and *B. subtilis*²⁰ biotin ligases. We then selected five additional

evolutionarily distant species whose biotin ligase genes were annotated in their sequenced genomes (*M. jannaschii*, *L. mesenteroides*, *T. cruzi*, *G. lamblia*, and *P. acnes*). The biotin ligase genes from these organisms were cloned into a bacterial expression vector and overexpressed in *E. coli*. All enzymes were obtained in reasonable yield and purity (Figure S1A), except for the human enzyme, which degraded significantly during isolation. Nevertheless, biotinylation activity was measurable for the human enzyme (*vide infra*), so we made use of this sample.

To assay the biotin ligases, we required a protein or peptide substrate. We and others previously found that the AP is recognized only by *E. coli* BirA, and not by biotin ligases from several other species.^{4,21} However, it has been observed that endogenous biotin acceptor proteins generally display cross-reactivity with biotin ligases from other species.²² We expressed and purified a domain of one of the endogenous biotin acceptor proteins of human biotin ligase, called p67.²³ By comparing the rates of biotin ligation to p67 for all the biotin ligases under identical conditions, we confirmed that all enzymes except the *G. lamblia* ligase recognized p67 (Figure S1B). The biotinylation rates measured under these conditions spanned a 700-fold range of activity.

Using p67 as substrate, we assayed the eight new biotin ligases with biotin and the seven analogs shown in Figure 1. In the assays, formation of probe-p67 conjugate was detected either by a change in the retention time of p67 on reverse-phase HPLC, or by a shift in p67 mobility on a native polyacrylamide gel.²⁴ None of the ligases incorporated probes **4-8** (Figure 1). However, we detected product in the reactions of *P. horikishii* biotin ligase (PhBL) with DTB-Az and PB (a mixture of *cis* and *trans* isomers), as well as yeast biotin ligase (yBL) with PB (Figure 1). Under identical conditions, the reaction of PB was much faster with yBL than with PhBL (data not shown), so we proceeded to determine which regioisomer of PB was preferred by yBL. The *cis* and *trans* isomers were separated by HPLC, then tested with yBL using the native gel shift assay. Using a reaction with biotin as a positive control for product formation and mobility shift, we observed no product with *trans*-PB, but complete conversion to product after 14 hours with *cis*-PB (Figure S2).

HPLC assays showed that both the PhBL-catalyzed ligation of DTB-Az and the yBL-catalyzed ligation of *cis*-PB were ATP- and enzyme-dependent (Figure 3). The product peaks were purified by HPLC and analyzed by mass spectrometry, which indicated that exactly one copy of DTB-Az or *cis*-PB had been ligated to each molecule of p67 by their respective enzymes (Figure S3). For comparison, the product of yBL-catalyzed biotinylation of p67 was also analyzed by mass spectrometry, and the product conjugate was clearly distinguishable from the p67-*cis*-PB conjugate. We also prepared a point mutant of p67, called p67(Ala), in which the lysine 61 modification site²³ was mutated to alanine. Mass spectrometry showed that p67(Ala) was not modified by either yBL or PhBL, demonstrating the site-specificity of these labeling reactions.

To further characterize these ligation reactions, we compared their kinetics to the kinetics of biotin ligation as catalyzed by the same enzymes (Figure S4). Under identical conditions with 1 mM probe (which was non-saturating for DTB-Az, data not shown), PhBL ligated DTB-Az to p67 at a rate of $1.34 \pm 0.11 \times 10^{-4} \mu\text{M s}^{-1}$, while biotin ligation occurred at a much faster rate of $0.20 \pm 0.02 \mu\text{M s}^{-1}$. We were able to measure the k_{cat} values for yBL ligation of *cis*-PB and biotin, because it was possible to saturate the yBL active site with 5 mM *cis*-PB (data not shown). We obtained a *cis*-PB ligation k_{cat} of $2.07 \pm 0.10 \times 10^{-2} \text{ s}^{-1}$, and a 14-fold higher k_{cat} for biotinylation of $0.28 \pm 0.04 \text{ s}^{-1}$. We attempted to accelerate the DTB-Az ligation kinetics by cloning one of *P. horikoshii*'s endogenous biotin acceptor proteins (the biotinyl domain of acetyl-CoA carboxylase), but the rate of DTB-Az ligation to this substrate was much slower than the rate with p67 (data not shown).

Finally, to demonstrate the utility of the azide ligation reaction for introduction of useful probes, we used PhBL to site-specifically attach DTB-Az to p67, and then we functionalized the introduced azide using a Staudinger ligation with a phosphine conjugate to the FLAG peptide (Figure 4).²⁵ Product was detected by immunoblotting with anti-FLAG antibody. Negative controls showed that ATP, PhBL, and DTB-Az were all required for the FLAG conjugation to p67.

In conclusion, by exploring biotin ligase enzymes from diverse species, we discovered that azide and alkyne derivatives of biotin are accepted in place of biotin by *P. horikoshii* and yeast biotin ligases, respectively. The crystal structures of PhBL in complex with biotin and the biotin adenylate ester have recently been described,¹⁶ but comparison to BirA crystal structures^{26,27} reveals a high degree of structural similarity in the biotin binding pockets, and no insight into the differential small-molecule substrate specificities of the two enzymes. No structure is yet available for the yeast biotin ligase.

Azides and alkynes are useful functional group handles that have been widely exploited in chemical biology for protein,^{25,28–30} DNA,^{31–33} sugar,^{9,34} small-molecule,³⁵ and virus tagging³⁶ *in vitro*, on the surface of living cells, and in living organisms. To truly harness the power of azide and alkyne-based bio-orthogonal ligation reactions, however, it is desirable to couple them with general methodology for site-specific introduction of azides and alkynes onto proteins or other biomolecules, particularly inside living cells. We note that both DTB-Az and *cis*-PB are more hydrophobic than biotin (based on HPLC retention times, data not shown), suggesting that they should be at least as membrane-permeable as biotin, which crosses mammalian cell membranes by passive diffusion at concentrations greater than 0.1 μ M.³⁷ In addition, at low concentrations, biotin is actively transported across the membrane by the sodium-dependent multivitamin transporter (SMVT).³⁸ The SMVT has been shown to interact with biotin analogs such as desthiobiotin,³⁸ so DTB-Az and *cis*-PB may be actively transported across mammalian membranes as well.

A future challenge will be to improve the kinetics of both ligation reactions, and to demonstrate their utility with peptide rather than protein substrates (for instance, with the yBL acceptor peptide that we recently discovered by phage display²¹). These efforts may be accomplished through a combination of rational mutagenesis and *in vitro* evolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Species of biotin ligase	Probes Tested							
	1	2	3	4	5	6	7	8
Human	+	-	-	-	-	-	-	-
<i>S. cerevisiae</i> (yBL)	+	-	+	-	-	-	-	-
<i>B. subtilis</i>	+	-	-	-	-	-	-	-
<i>P. acnes</i>	+	-	-	-	-	-	-	-
<i>L. mesenteroides</i>	+	-	-	-	-	-	-	-
<i>T. cruzi</i>	+	-	-	-	-	-	-	-
<i>M. jannaschii</i>	+	-	-	-	-	-	-	-
<i>P. horikoshii</i> (PhBL)	+	+	+	-	-	-	-	-

Figure 1.

Screening biotin ligases against biotin analogs. Top, structures of biotin analogs used in this study. Bottom, table showing the hits from screens. 1 μ M of each enzyme was incubated with 1 mM probe and 100 μ M p67 acceptor protein for 14 hours at 30 °C. Formation of product (indicated by “+”) was detected by HPLC or native gel shift assay. For screening, probe **3** (PB) was provided as a mixture of *cis* and *trans* isomers.

Figure 2.

Synthetic routes to (A) desthiobiotin azide and (B) *cis*-propargyl biotin. MeOH, methanol; Me, methyl; MsCl, mesyl chloride; AcCl, acetyl chloride; TFAA, trifluoroacetic anhydride; TEA, triethylamine.

Figure 3. HPLC detection of (A) DTB-Az and (B) *cis*-PB ligation to p67 acceptor protein by PhBL and yBL, respectively. Negative controls are shown with ATP or enzyme omitted. The biotin-p67 conjugate has a shorter retention time than the DTB-Az or *cis*-PB conjugates. The starred peaks were collected and analyzed by mass spectrometry in a separate experiment (Figure S3).

Figure 4.

Functionalization of site-specifically ligated DTB-Az by Staudinger ligation. PhBL was used to ligate DTB-Az to p67 acceptor protein. The introduced azide was then functionalized with phosphine-FLAG.²⁵ The FLAG epitope was detected with anti-FLAG antibody staining (α -FLAG). Negative controls show omission of ligase, DTB-Az, or ATP from the reactions (lanes 2–4). Ponceau S staining of the same blot (right) demonstrates equal protein loading in all lanes. Unmodified p67 has a slightly lower molecular weight than FLAG-conjugated p67.