Capture-Tag-Release: A Strategy for Small Molecule Labeling of Native Enzymes

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A strategy for labeling native enzymes in a manner that preserves their activity is reported: capture–tag–release (CTR). Key to this approach is the small molecule CTR probe that contains an enzyme inhibitor, benzophenone crosslinker, and aryl phosphine ester. After UV-derived capture of the enzyme, addition of an azide-containing tag triggers a Staudinger ligation that labels the enzyme. A further consequence of the Staudinger ligation is fragmentation of the CTR probe, thus releasing the inhibitor and restoring enzymatic activity. As a proof-of-principle, the CTR strategy was applied to the β-galactosidase. The enzyme was efficiently labeled with biotin, and the kinetic data for the biotinylated enzyme were comparable to those for unlabeled β-galactosidase. The CTR probe exhibits excellent targeting specificity, as it selectively labeled β-galactosidase in a complex protein mixture.

The ability to selectively label proteins with small molecules is important for the study of biological systems. Commonly, a protein of interest is genetically engineered to contain an unnatural amino acid,[1] peptide sequence,[2] or domain[3] that reacts with a small molecule tag. Although such labeling strategies typically enjoy excellent selectivity, they cannot be used to profile and study native proteins. Consequently, a number of reactions have been developed to label canonical amino acids; however, discriminating between identical side chains both within a target protein and on different proteins remains challenging.[4] Affinity-based strategies have significantly improved the labeling selectivity for a range of native proteins.[5] In the case of enzymes, exquisite selectivity has been achieved by activity-based protein profiling.[6] Although this is a powerful proteomics tool, the use of irreversible inhibitors precludes subsequent studies of enzyme activity. Consequently, few methods exist to label a native enzyme in a manner that preserves its function.[7]

We envisioned that the Staudinger ligation (pioneered by Bertozzi and co-workers)[8] could be applied to the challenge of native-enzyme labeling. As its name implies, this transformation is also a fragmentation, ejecting molecular nitrogen and cleaving the ester C–O σ-bond. Mechanistic studies have shown that the ester can accommodate a range of linear and branched substituents without affecting the overall rate of ligation.[9] yet few applications of the Staudinger ligation utilize the ester as a site for chemical modification.[10] Although traceless versions of the Staudinger ligation have been developed, they generally involve the fragmentation of a thiocysteine to expel the original phosphine.[11]

Here we report a strategy ("capture–tag–release", CTR) for labeling native enzymes, by exploiting the unique ester fragmentation properties of the Staudinger ligation. Key to the approach is the small-molecule CTR probe, which contains an enzyme inhibitor and a benzophenone crosslinker connected by an aryl phosphine ester (Scheme 1). The probe derives its targeting specificity from the enzyme inhibitor. Once the enzyme-inhibitor complex is formed, UV-irradiation triggers benzophenone crosslinking, thereby covalently capturing the enzyme (Scheme 1, step 1). Treating this adduct with an azide-containing tag (for example, biotin) then initiates the Staudinger ligation, thereby labeling the enzyme. Step 3) A consequence of the Staudinger ligation is fragmentation of the CTR probe, thus releasing the inhibitor and restoring enzymatic activity.

Scheme 1. Capture-tag-release (CTR) strategy for small-molecule labeling of native enzymes. Step 1) The CTR probe forms an enzyme-inhibitor (E·I) complex, and the benzophenone covalently captures the enzyme upon UV irradiation. Step 2) Treatment with an azide-containing tag triggers a Staudinger ligation, thereby labeling the enzyme. Step 3) A consequence of the Staudinger ligation is fragmentation of the CTR probe, thus releasing the inhibitor and restoring enzymatic activity.

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sylation of lactose into other disaccharides. Our modular synthesis began with acetylated thiol 1, based on the competitive inhibitor isopropyl-thio-β-D-galactopyranoside (IPTG) (Scheme 2). The thiol was alkylated with poly(ethylene glycol) bromide linkers of varying lengths to afford alcohols 2–4. Multiple linker lengths allow the benzophenone to sample different surface amino acids in the capture step. Synthesis of the crosslinker terminus began by coupling amine 5 to carboxylic acid 6. Subsequent azide reduction and coupling of amine 7 with activated N-hydroxysuccinimide (NHS) ester 8 afforded methyl ester 9. This compound (lacking a competitive inhibitor) served as a negative control in our crosslinking studies. Saponification of 9 yielded the corresponding carboxylic acid, which was then coupled with alcohols 2, 3, and 4 by using EDC-HCl and DMAP. Finally, chemoselective removal of the aca-

tate protecting groups in the presence of the benzoate linker was accomplished with guanidine-NaCl to afford CTR probes 13–15.

Having synthesized this small panel of CTR probes, we assessed binding to β-gal. Enzyme activity was measured under steady-state conditions across a range of probe concentration (Figure 1). The apparent Michaelis–Menten constant \( K_{\text{m,app}} \) increased as a function of inhibitor concentration while the maximum rate \( (V_{\text{max}}) \) did not (Figure 1 and Figure S1 in the Supporting Information), consistent with the CTR probes functioning as competitive inhibitors. This is expected as the probes share the thiogalactose motif of IPTG, which is a competitive inhibitor of β-gal. From these data, we also calculated the dissociation constant \( (K) \) for each inhibitor. Replacing the branched isopropyl side chain of IPTG with the linear aryl ester of the CTR probe resulted in a tenfold improvement in \( K \) for β-gal (Table 1).

To test the labeling efficiency of our CTR probes, we chose a biotin tag, because of its broad utility and straightforward detection. Beginning with the capture step, the purified enzyme was incubated with 13, 14, or 15 and irradiated at 350 nm (30 min). The tag and release steps were accomplished by adding biotin azide (commercially available) and incubating at 30 ºC (4 h). The samples were resolved by gel electrophoresis, and biotin was visualized by western blotting with a streptavidin-HRP conjugate (Figure 2). Quantitative densitometry (biotinylated internal standard in lane 12) revealed that 13–15 biotinylated β-gal in a dose-dependent fashion. Gratifyingly, the shortest probe (13) labeled 95% of the enzyme at the highest concentration tested (lane 4). Control experiments confirmed that both UV irradiation and the CTR probe are required.
Importantly, removing the thiohistone deacetylase (HDAC) from HDAC1 (Figure 3 and Table S1). It is unlikely that this rescue in function was due to ester hydrolysis, as the CTR probe demonstrated excellent stability in aqueous buffer (Figure S4). Collectively, these data indicate that despite having a biotin tag near the active site, the enzyme behaved comparably to the native species.

Finally, we investigated the targeting specificity of 13. First, a competition experiment was performed by using 13 and increasing amounts of the competitive inhibitor IPTG. We observed a dose-dependent decrease in β-galactosidase activity at key steps in the CTR sequence. The enzyme and 13 were incubated, crosslinked, and free small molecules were removed by gel filtration. Activity was measured as described above. The enzyme displayed extremely low activity, consistent with the probe blocking the active site (Figure 3). However, upon addition of biotin azide, the CTR probe fragmented and released its inhibitor. The biotinylated enzyme exhibited impressive turnover (91% compared to unlabeled enzyme) with only a minor increase (16%) in $K_m$ (Figure 3 and Table S1).

In summary, the developed capture–tag–release (CTR) strategy helped to address the enduring challenge of native-enzyme labeling. Our approach exploits an underutilized fragment pathway in the Staudinger ligation, in order to eject the targeting inhibitor and restore enzyme activity. We show that a CTR probe can efficiently biotinylate β-galactosidase, and that this labeling does not significantly affect its function. Moreover, the targeting specificity of the CTR probe was demonstrated by competition with the known inhibitor IPTG and by selectively labeling β-gal in a complex protein mixture (Figure 4B). Of the seven proteins present, only β-gal was biotinylated (lane 4). Labeling specificity was again confirmed by competition with excess IPTG (lane 5).

In summary, the developed capture–tag–release (CTR) strategy helped to address the enduring challenge of native-enzyme labeling. Our approach exploits an underutilized fragment pathway in the Staudinger ligation, in order to eject the targeting inhibitor and restore enzyme activity. We show that a CTR probe can efficiently biotinylate β-galactosidase, and that this labeling does not significantly affect its function. Moreover, the targeting specificity of the CTR probe was demonstrated by competition with the known inhibitor IPTG and by selectively labeling β-gal in a complex protein mixture. There are a growing number of potent and selectivesmall-molecule ligands for enzymes (e.g., kinases,[18] histone deacetylases,[19] methyltransferases and demethylases),[20] as well as protein domains (e.g., bromodomains).[21] These targeting ligands could be easily incorporated into the existing modular
synthesis of CTR probes, thus significantly broadening their scope. We believe that CTR has the potential to be a general strategy for protein labeling and a useful tool for chemical biology.

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