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Traceless Affinity Labeling of Endogenous Proteins for Functional Analysis in Living Cells

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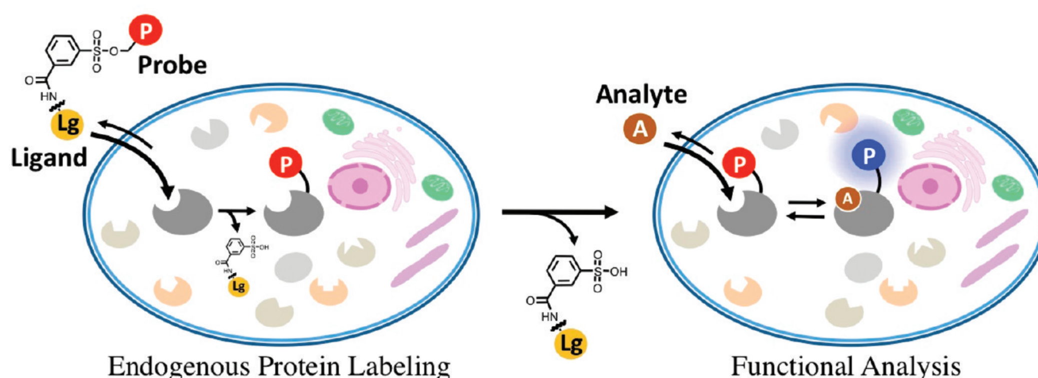

Protein labeling and imaging techniques have provided tremendous opportunities to study the structure, function, dynamics, and localization of individual proteins in the complex environment of living cells. Molecular biology-based approaches, such as GFP-fusion tags and monoclonal antibodies, have served as important tools for the visualization of individual proteins in cells. Although these techniques continue to be valuable for live cell imaging, they have a number of limitations that have only been addressed by recent progress in chemistry-based approaches. These chemical approaches benefit greatly from the smaller probe sizes that should result in fewer perturbations to proteins and to biological systems as a whole. Despite the research in this area, so far none of these labeling techniques permit labeling and imaging of selected endogenous proteins in living cells.

Researchers have widely used affinity labeling, in which the protein of interest is labeled by a reactive group attached to a ligand, to identify and characterize proteins. Since the first report of affinity labeling in the early 1960s, efforts to fine-tune the chemical structures of both the reactive group and ligand have led to protein labeling with excellent target selectivity in the whole proteome of living cells. Although the chemical probes used for affinity labeling generally inactivate target proteins, this strategy holds promise as a valuable tool for the labeling and imaging of endogenous proteins in living cells and by extension in living animals.

In this Account, we summarize traceless affinity labeling, a technique explored mainly in our laboratory. In our overview of the different labeling techniques, we emphasize the challenge of designing chemical probes that allow for dissociation of the affinity module (often a ligand) after the labeling reaction so that the labeled protein retains its native function. This feature distinguishes the traceless labeling approach from the traditional affinity labeling method and allows for real-time monitoring of protein activity. With the high target specificity and biocompatibility of this technique, we have achieved individual labeling and imaging of endogenously expressed proteins in samples of high biological complexity. We also highlight applications in which our current approach enabled the monitoring of important biological events, such as ligand binding, in living cells. These novel chemical labeling techniques are expected to provide a molecular toolbox for studying a wide variety of proteins and beyond in living cells.

1. Introduction

Over the past years, the main focus of proteome research has been gradually shifting from understanding how individual proteins work in test tubes to how proteins function in the context of native environments, that is, in cells or in animal bodies. Protein labeling and imaging techniques have been one of the most valuable methods to study structure, dynamics, function, and cellular localization of

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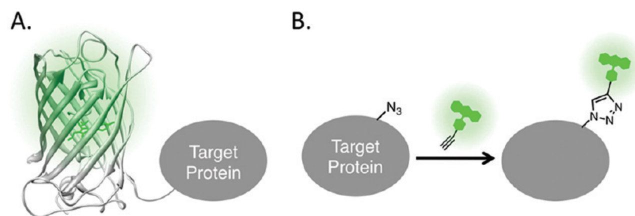

FIGURE 1. Schematic representations of (A) molecular biology-based and (B) chemistry-based protein labeling methods.

proteins. Molecular biology-based protein labeling methods such as GFP-fusion tags and monoclonal antibodies have served as powerful tools to visualize many types of proteins (Figure 1A).[1,2] While these techniques allow for real-time monitoring of individual proteins in living organisms, their relatively large size or undesired protein–protein interactions often prevents sophisticated biophysical measurements.[3] Synthetic chemistry based approaches, on the other hand, may overcome such limitations, because much smaller synthetic probes will likely result in no or minimal perturbations to the structure and function of labeled proteins (Figure 1B). A breakthrough for chemical probes came with the establishment of bioorthogonal chemistry.[4] This approach involves metabolic or genetic incorporation of chemical entities that do not usually exist in living systems, followed by the addition of chemical probes that react specifically with the noncanonically introduced chemical handle (Figure 1B). Although undeniably powerful and highly selective, this approach involves genetic modifications or metabolic uptake of labeled biomolecules as prerequisites, thereby limiting their applications. Thus, methods for selective labeling and imaging of endogenously expressed proteins, namely native proteins, in their native habitats are in high demand.[5] The methodologies for such purposes would have features of (1) no prelabeling processes required, (2) no interference with the protein function, and (3) high selectivity toward target proteins in samples of high biological complexity.

One way to meet such criteria is to use affinity labeling. The term "affinity labeling" was introduced by Singer et al. in the early 1960s, although related investigations had been independently reported from several groups almost at the same time.[6−8] The concept was to place a chemically or photochemically reactive handle on the substrate-like or ligand-like compound (Figure 2A) and covalently modify the target proteins upon specific ligand recognition (Figure 2B). In the early days, this (photo)affinity labeling with radioactive ligands was widely used for the identification of various proteins, mostly receptors and transporters.[9,10]

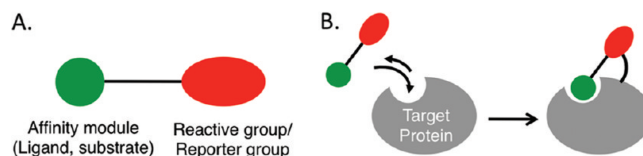

FIGURE 2. Affinity-based protein labeling: (A) the general design of affinity probes and (B) its reaction scheme.

When modern mass spectroscopy (MS) techniques became available, this approach provided additional information regarding the structure and function of target proteins, such as the identification of exact amino acid residues in active sites and the examination of stoichiometry and site specificity of biomolecular interactions.[11,12] More recently, Cravatt et al. have made substantial improvements on the prototype affinity probes and developed a way to directly evaluate protein activity, rather than abundance, in whole proteomes.[13,14] This method, termed activity-based protein profiling (ABPP) enables the characterization of more than a dozen enzymes, including (metallo)proteases, kinases, phosphatases, glycosidases, and oxidoreductases in their active states.[15] However, the chemical probes used in these approaches usually inactivate the target proteins, limiting valuable potential applications, such as real-time monitoring of protein activity *in vivo*.

From the viewpoint of protein engineering, we became particularly interested in affinity labeling and sought to develop methods for monitoring protein activity in living organisms. Only a decade ago, we reported the first example of affinity labeling, in which the protein activity was fully conserved in test tubes.[16] After several years of "trial and error", we subsequently developed new chemical methods that allowed target protein labeling and the monitoring of the activity of these labeled proteins in living cells.[17,18] In this Account, we summarize the affinity-based native protein labeling we have explored over the past decade.

2. Traceless Affinity Labeling of Native Proteins

As mentioned above, affinity-driven protein labeling offers a highly selective means of protein modification with synthetic probes. The key to our strategy is that the ligand is removed after labeling such that the labeled protein retains its native function, thereby allowing for real-time monitoring of protein activity. Our labeling methods are roughly divided into two: an exchange/cleavage reaction type and a catalyst tethering type (Figure 3). The former reaction proceeds via three steps: (1) recognition of the ligand part of the labeling

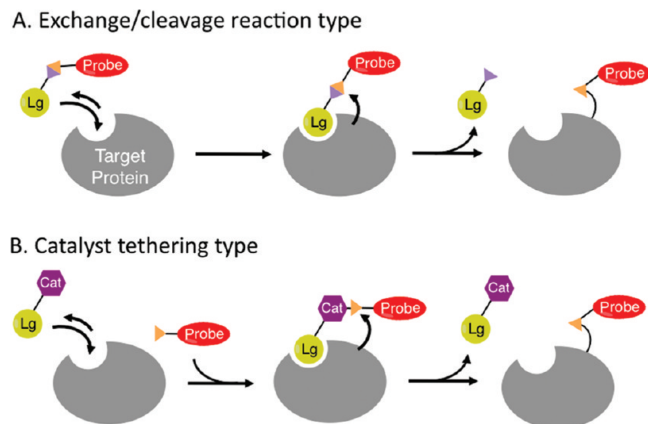

FIGURE 3. General concept of traceless affinity-based protein labeling: (A) the exchange/cleavage reaction type and (B) the catalyst tethering type.

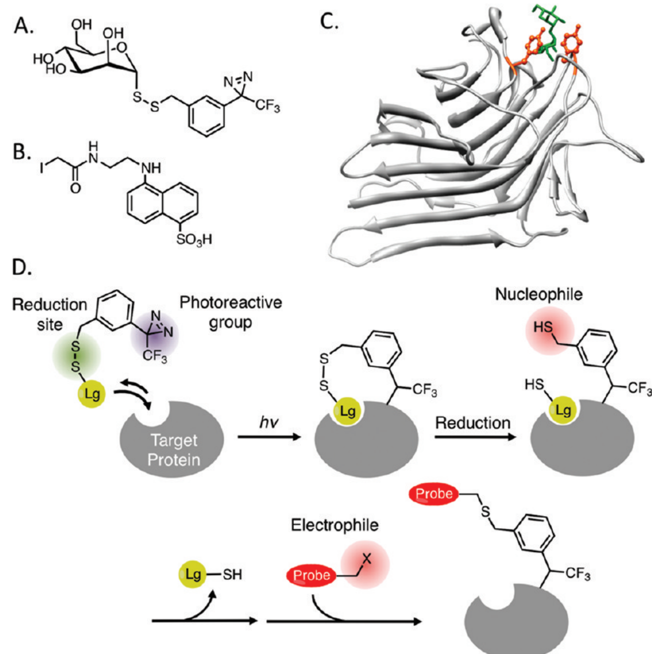

FIGURE 4. Chemical structure of P-PALM reagents, (A) labeling reagent and (B) fluorescent probe, and (C) crystal structure of ConA. The orange residues represent Tyr100 and Tyr12 labeled by P-PALM, and green represents the mannobiose (PDB ID 1I3H). (D) Also shown is the general reaction scheme of P-PALM.

reagent; (2) covalent attachment to the target protein; (3) concurrent cleavage or subsequent exchange of the ligand moiety (Figure 3A). The catalyst tethering reaction also proceeds via three steps: (1) recognition of the ligand tethered to the catalyst; (2) activation and covalent attachment of the probe; (3) dissociation of the ligand (Figure 3B). In both cases, the labeling reaction is driven by the "proximity effect", that is, reactive groups are brought to or formed near the ligand-binding site and only specific amino acid residues near the ligand-binding site are modified.

2.1. The First Generation of Traceless Affinity Labeling: Post-photoaffinity Labeling Modification (P-PALM). We have employed photoreactive groups as reactive modules in our first generation affinity labeling, termed post-photoaffinity labeling modification (P-PALM).[16] As our initial target, concanavalin A (ConA), a plant lectin, was used (Figure 4C). We designed the labeling reagent to contain three functionalities: (1) a high-affinity protein ligand (α-D-mannoside or α-D-glucoside); (2) a photoreactive group (diazirine); (3) a cleavage site (disulfide) that can generate a chemoselective reaction site (nucleophilic thiol group) upon chemical reduction (Figure 4A). The saccharide moiety of this labeling reagent binds to ConA, which brings the photoreactive group nearby the ligand-binding site of the protein, that is, proximity effect (Figure 4D). UV irradiation of this complex results in covalent attachment of the reagent. After gel filtration and affinity chromatography, the labeled ConA was treated with DTT to generate a mercaptobenzyl group on ConA, which is then modified with a series of fluorescent probes bearing electrophiles, such as iodoacetamide and maleimide groups (Figure 4B,D).[16,19,20] Despite the highly reactive nature of the photoreactive groups, we revealed that the labeling reaction occurs exclusively on tyrosine residues located proximal to the saccharide binding pocket (Tyr100 or Tyr12) (Figure 4C).

Monitoring the concentration level of metabolites, such as saccharides, is of particular importance in biological research and diagnosis. Although FRET-based biosensors have been developed for some sugars, most rely on drastic conformational changes of the proteins upon ligand binding. When ConA was modified with an environmentally sensitive fluorescent dansyl probe (DANS-ConA), the DANS-ConA functioned as a fluorescence biosensor for the sugar with a smaller conformational change occurring to the ConA scaffold. The spectral change upon addition of methyl-α-mannoside gave a binding constant that was identical to that determined for native ConA by isothermal titration calorimetry.[21] Further, the same titration experiments with other saccharides gave binding constants similar to those obtained for native ConA, indicating that the fluorescent probe was successfully introduced into ConA without compromising its native function.

2.2. The Second Generation Traceless Affinity Labeling: Postaffinity Labeling Modification (P-ALM). Regardless of the partial success of P-PALM, this method is not applicable to proteins containing cysteine residues, which may potentially be modified by the thiol chemistry. Additionally, the

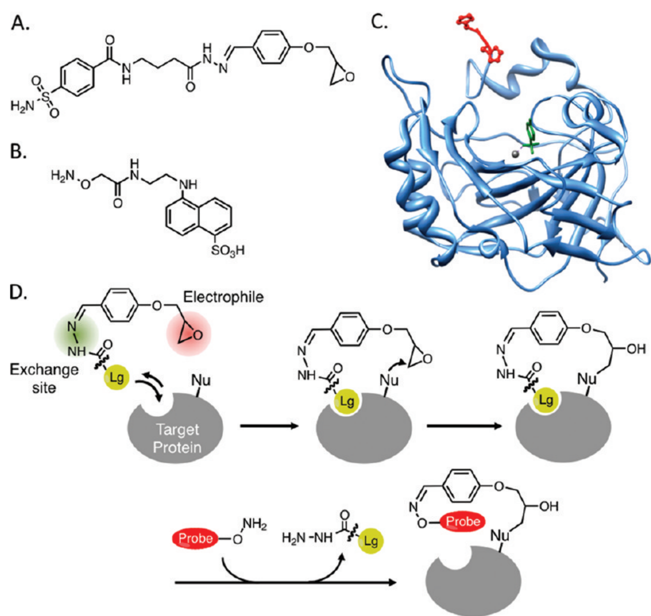

FIGURE 5. Chemical structure of P-ALM reagents, (A) labeling reagent and (B) fluorescent probe, and (C) crystal structure of hCAII. Red colored residues represent His3 and His4 labeled by P-ALM, and green represents an inhibitor 4-fluorobenzenesulfonamide (PDB ID 1IF4). (D) Also shown is the general reaction scheme of P-ALM.

approach requires several purification steps in order to remove the ligand, excess reagents, and byproducts, making the method difficult and not practical in the presence of other proteins. In order to address these issues, we have proposed an improved method, called postaffinity labeling modification (P-ALM).[22,23] This strategy involves the affinity-based modification of a target protein with a unique reactive handle, followed by selective transformation of this handle by a bioorthogonal reaction to introduce a variety of probes into the target protein. Human carbonic anhydrase II (hCAII) was employed as a proof-of-principle model (Figure 5C), and a labeling reagent was designed that has an affinity ligand (benzene sulfonamide) and an electrophilic reactive group (epoxide) linked by an exchangeable unit (hydrazone) (Figure 5A). As with P-PALM, the ligand moiety of the labeling reagents brings the epoxide group nearby the surface of the hCA active site, which facilitates the nucleophilic attack of a protein amino acid to the electrophile, that is, epoxide without light irradiation (Figure 5D).[24] Subsequent addition of probes containing aminooxy or hydrazine groups allows for the dissociation of the ligand moiety and incorporation of the probes to the protein via the hydrazone/oxime exchange reaction with almost 100% yield (Figure 5D).

It is interesting that the labeling occurred exclusively on two histidine residues (His3 and His4) of hCAII located on a protein surface near the active site (Figure 5C). Most

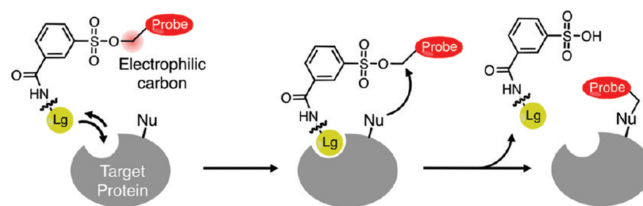

FIGURE 6. General reaction scheme of LDT chemistry. Lg and Nu denote an affinity ligand and a nucleophilic residue on the protein surface, respectively.

importantly, the enzyme activity assay of the labeled hCAII using the conventional hydrolysis reaction of *p*-nitrophenyl acetate showed that the labeled hCAII retains its activity with both K_m and k_{cat} parameters similar to those of native hCAII.

2.3. The Third Generation Traceless Affinity Labeling: Ligand-Directed Tosyl (LDT) Chemistry. While our first- and second-generation affinity labeling methods allowed for labeling of native proteins without compromising their native functions, their applications remained limited only to *in vitro* systems owing to the multistep labeling protocol used and the low bioorthogonality. By unifying the covalent bond formation and ligand cleavage, we have developed a new labeling method, called ligand-directed tosyl (LDT) chemistry, in which the phenyl sulfonate (tosyl) linker plays roles both as a linker between the ligand and the reactive group and as a good leaving group (Figure 6).[17,25] The S_N2-type reaction between the tosyl ester moiety and a nucleophilic amino acid residue results in the release of the ligand moiety upon the labeling reaction, and thus the protein retains activity.

The utility of LDT chemistry was first demonstrated by hCAII modification. LDT reagents were synthesized that have benzenesulfonamide and synthetic probes, a fluorophore (Dc, 7-dimetylaminocoumarin) or biotin tag, which are connected through tosylate (Figure 7A). After 48 h of incubation at 37 °C using the Dc-type labeling reagent in a test tube, hCAII was labeled with Dc with a relatively high yield (~75%). Further, the labeling experiment in the presence of other proteins showed that only hCAII was labeled, suggesting that the labeling reaction proceeds with high protein selectivity. The peptide mapping analysis identified that His3, which is located proximal to the active site and was also labeled by P-ALM, was predominantly labeled by LDT chemistry (Figure 5C). As with P-ALM, the labeled hCAII displayed enzyme activity identical to that of native hCAII.

Given the high selectivity achieved by the current approach, we next attempted labeling of endogenously expressed hCA in living cells. We incubated human red blood cells (hRBCs), which

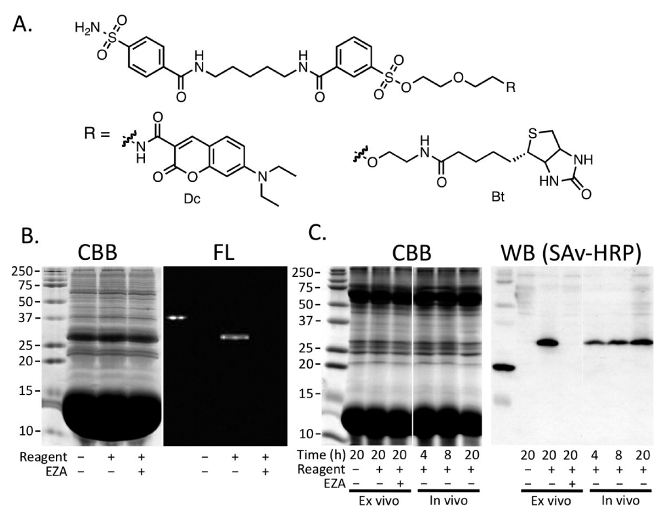

FIGURE 7. LDT reagents used for hCA labeling: (A) 7-dimethylamino-coumarin (Dc) and the biotin-tag (Bt) type. CA-selective labeling in (B) RBCs and (C) in a living mouse. The labeling reaction was detected by SDS–PAGE combined with CBB staining, fluorescence imaging (FL), and Western blotting (WB).

stably express hCAI and II, in the presence of the Dc-type labeling reagent for 48 h, during which no hemolysis was observed. The SDS–PAGE after lysis exhibits a single fluorescent band corresponding to endogenous hCA (Figure 7B). Importantly, when the same reaction was conducted in the presence of an inhibitor, 6-ethoxy-benzothiazole sulfonamide (EZA), the hCA labeling was not observed, suggesting that selective labeling was achieved by the affinity-based reaction (Figure 7B). Encouraged by these results, we took one step further, that is, hCA labeling in an animal body. The biotin-type labeling reagent was intravenously injected into Slc:ICR mice, and after corresponding hours blood samples were taken from the tail vein and analyzed by Western blotting. Remarkably, a single band was detected by a streptavidin–horseradish peroxidase conjugate (SAv–HRP), which was also identified by an anti-mouse CA antibody, suggesting that CA-selective labeling occurs in a living body (Figure 7C). These results clearly demonstrated the validity of the LDT chemistry in the context of whole proteomes.

The characteristics of LDT chemistry demonstrated to date are (1) sufficient stability in biological conditions, (2) a one-step labeling process, (3) no or minimal perturbation of the target protein, and (4) high selectivity toward target proteins in whole proteomes. Furthermore, the current modular approach is applicable to, in principle, any endogenous protein by simply switching the ligand moiety. We have successfully labeled FKBP12, Src homology 2 (SH2) domain, and congerin II (CongII) using LDT reagents containing the synthetic analog of FK506, peptide, and lactose ligands.[17]

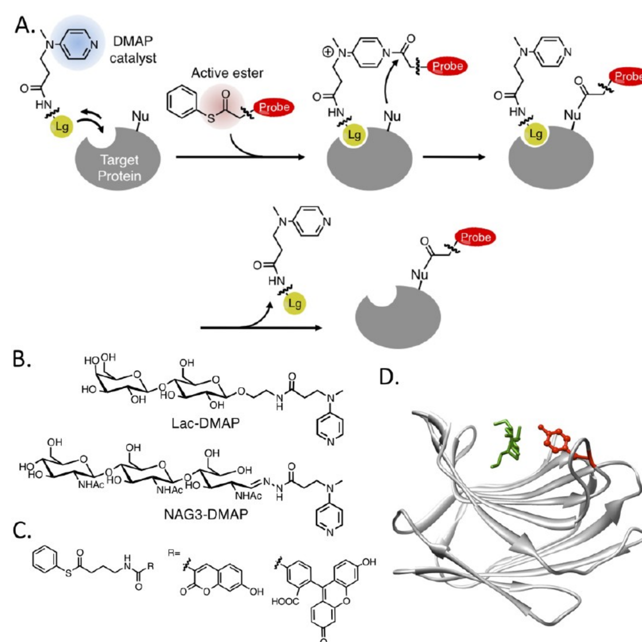

FIGURE 8. (A) General reaction scheme of AGD chemistry. Chemical structure of AGD reagents, (B) ligand-tethered DMAP catalysts and (C) acyl donors, and (D) crystal structure of CongII. The red color residue is Tyr51 labeled by AGD, and the green structure is lactose (PDB ID 1IS4).

More recently, other groups have elegantly utilized LDT chemistry for selective protein labeling, such as heat shock cognate 70 (Hsc70), a molecular chaperone, and 14-3-3 proteins.[26,27]

2.4. Alternative Traceless Affinity Labeling: Affinity-Guided DMAP (AGD) Chemistry. An alternative approach to protein labeling involves the use of a catalyst to facilitate the chemical modification of target proteins (Figure 3B).[18,28] Francis and co-workers have recently demonstrated the use of transition metal catalysts as powerful tools for protein modification with excellent chemoselectivity in the test tube.[29,30] In addition to transition metal catalysts, organocatalysts should be useful for protein labeling. We have developed a new method, termed affinity-guided DMAP (4-dimethylaminopyridine) chemistry or AGD chemistry. DMAP is a commonly used catalyst for acyl transfer reactions, which can activate and transfer an ester to a nucleophile.[31] Our strategy is depicted in Figure 8A. We designed an affinity ligand tethered to the DMAP catalyst, which, in the presence of appropriate acyl donors, facilitates the acyl transfer reaction to a nucleophilic amino acid residue near the active site of target proteins.

To test our idea, we used CongII, an animal lectin with high affinity for lactose and LacNAc (Figure 8D). We prepared lactose- and NAG3-tethered DMAP catalysts and acyl donors (Figure 8B,C). During optimization of the labeling reagents,

we found that the structure of the acyl donors should be carefully chosen in both the carboxylic ester part (acyl group) and the thiol part (leaving group) in order to achieve efficient and selective protein labeling. The acyl donor containing benzyl thioester is not sufficiently reactive to be activated by DMAP, as compared with the ones containing a thiophenyl ester. On the other hand, among the thiophenyl esters, the acyl donor with an α-amino acid scaffold was too reactive, causing a considerable amount of nonspecific acylation and rapid hydrolysis. We found that an acyl donor with a γ-amino acid exhibits enhanced specificity with the least number and amount of side reactions (Figure 8C). We then carried out labeling reactions of CongII using Lac-DMAP with the optimal acyl donors at 25 °C in 35% yield. The labeling occurred on Tyr51, presumably on its OH group, proximal to the sugar binding pocket (Figure 8D). The fluorescence titration experiments with various saccharides showed that the fluorescein-labeled CongII exhibits sugar-binding ability similar to that of the native CongII, as with other labeling techniques.

Like LDT, the AGD-based approach greatly benefits from its modular design. By simply switching the selectivity module (ligand), one can easily alter the target protein. Currently, we have designed a series of ligand-tethered DMAP catalysts specific to lectins (CongII, ConA, wheat germ agglutinin), the SH2 domain, and FKBP12 (Figure 9A) and have demonstrated the high target specificity in a bacterial cell lysate and an animal tissue extract.[18,28]

To our surprise, we recently discovered that the labeling reaction can be greatly accelerated by increasing the number of DMAP groups in the ligand–catalyst complex (Figure 9B).[18] The initial rate of CongII labeling was accelerated by 3.6- and 4.6-fold when the number of DMAP moieties was increased from one to two and three, respectively (Figure 9C). The effect was even more pronounced with the SH2 domain and FKBP12, where the rates were accelerated more than 10-fold with tri-DMAP catalysts compared with the mono-DMAP counterparts (Figure 9C). Interestingly, we have found that the DMAP moiety of the multivalent DMAP catalysts acts not only as an activator but also as a base to accelerate the acyl transfer reactions. Thus, with a multivalent DMAP unit, the chemical acylation reaction of the target protein was significantly accelerated, making the reaction efficient and more suitable to *in vivo* protein labeling. An example of this reaction is detailed in section 3.2.

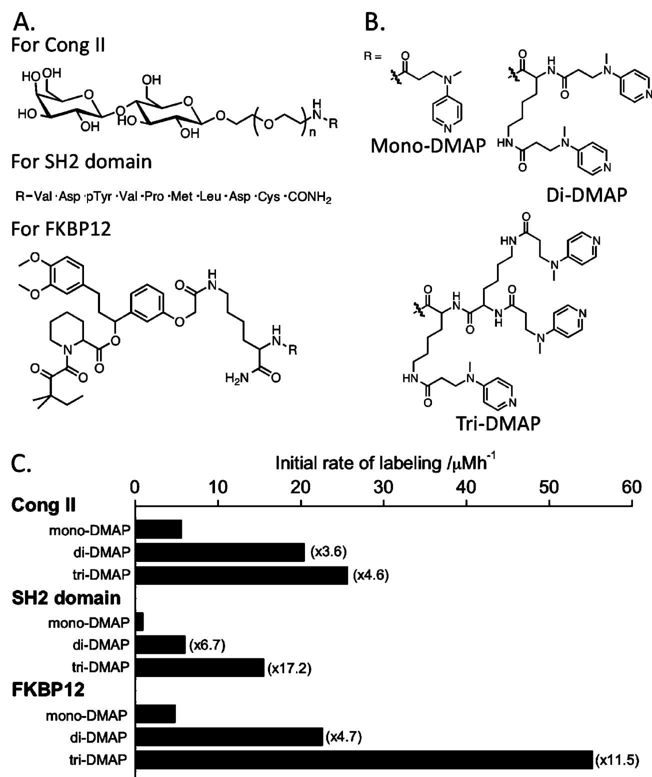

FIGURE 9. (A) Chemical structure of AGD catalysts for CongII, the SH2 domain, and FKBP12 with (B) mono-, di-, and tri-DMAP, and (C) initial rates of the labeling reactions obtained for CongII, the SH2 domain, and FKBP12 with mono-, di-, and tri-DMAP catalysts.

3. Applications of Traceless Affinity Labeling

The applications of our affinity labeling were initially limited to *in vitro*. However, we accomplished extending these methods to crude protein mixtures such as cell lysates, living cells, and ultimately to living animals. Moreover, because of the modular strategy, chemical probes are not limited to fluorescence probes or biotin tags and can be creatively designed for a variety of applications. Here we describe a few examples of the many applications of the affinity labeling and engineering of native proteins.

3.1. *In Vitro* Applications . 3.1.1. Biosensors for Oligosaccharides Using a Lectin Scaffold. In a previous section, we described that ConA labeled with an environmentally sensitive fluorophore worked as a fluorescence-based biosensor for various saccharides. The strategy was rather unique, because the biosensor did not require drastic structural changes upon sugar binding. Using P-PALM, we further incorporated a synthetic receptor into the ConA scaffold with the aim that the affinity for certain sugars can be enhanced. A phenylboronic acid (PBA)-based fluorescent chemosensor (APET) whose signal switches on upon recognition of 1,2- and 1,3-diols due to the PET mechanism[32] was attached for construction of a semisynthetic fluorescent biosensor (APET-ConA) (Figure 10A,B).[33] The cooperative binding by PBA and ConA afforded an improved selectivity

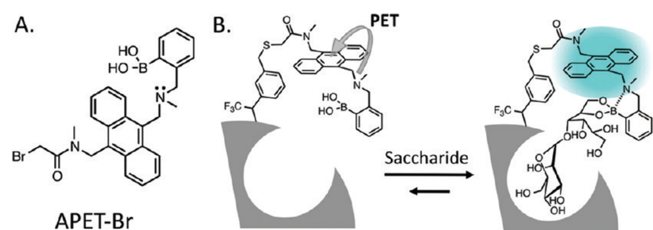

FIGURE 10. (A) Chemical structure of APET-Br and (B) schematic representation of the PET mechanism of cooperative saccharide binding and sensing by APET-ConA.

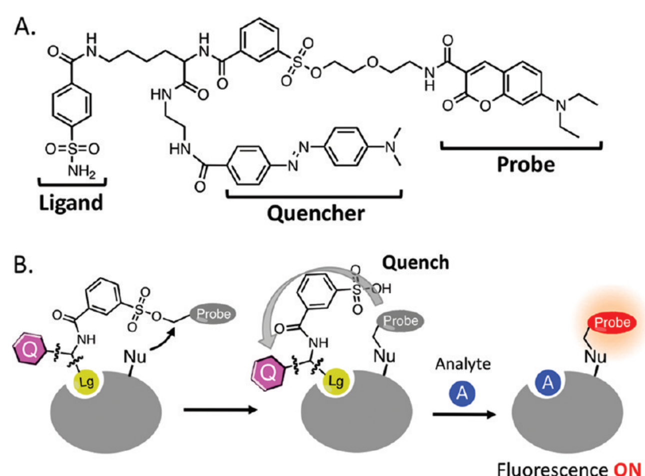

FIGURE 11. (A) Chemical structure of the quenched LDT (Q-LDT) reagent and (B) the schematic representation of the Q-LDT-based biosensor.

and binding affinity for specific oligosaccharides over monosaccharides. While lectins have been used to analyze certain carbonhydrates, their relatively low selectivity and binding affinity prevents robust and accurate high-throughput assays of sugars. This example clearly represents coupling of artificial sugar recognition sites with native lectins as a promising strategy to enhance the binding affinity for certain sugars.

3.1.2. Turn-on Fluorescence Biosensor. LDT chemistry is quite unique in that the ligand moiety is cleaved upon covalent bond formation between the reagent and the target protein. Given the features of the current approach, we sought to develop a turn-on type fluorescence biosensor by incorporating a fluorescence quencher in the dissociating ligand part.[25] Our strategy is depicted in Figure 11. We designed a quencher-tethered LDT (Q-LDT) reagent containing both a fluorescent probe and quencher within one molecule such that the reagent itself is weakly fluorescent (Figure 11A). Upon the labeling reaction, the ligand moiety is cleaved off but still remains within the ligand-binding site of the protein, and therefore the fluorescence is largely quenched (Figure 11B). However, addition of exogenous ligand causes displacement of the quencher-tethered ligand, resulting in fluorescence recovery (Figure 11B). This bimolecular fluorescence quenching and recovery (BFQR)-based approach has been applied for ligand binding assays of hCAII and the SH2 domain both in purified protein solutions

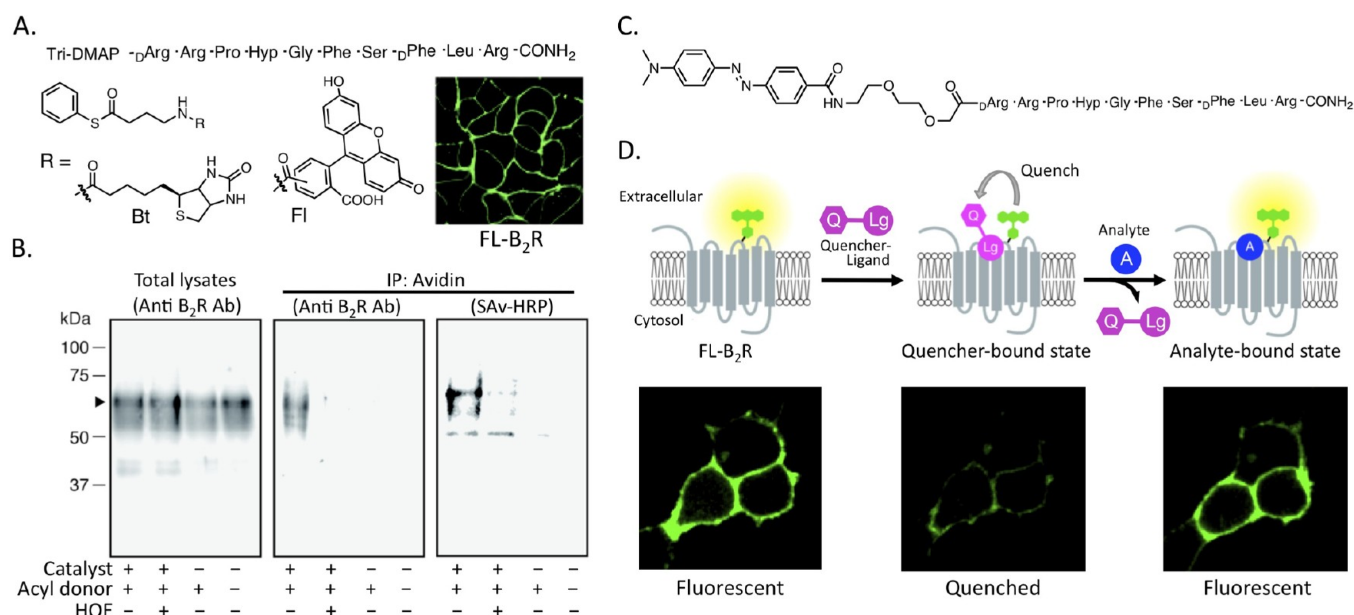

FIGURE 12. (A) Chemical structure of AGD reagents for B_2R and fluorescein-labeled B_2R on HEK293 cells, (B) Western blotting analysis of biotin-labeled B_2R on HEK 293, (C) structure of the quencher 4-dimethylaminophenolazobenzene-4-carboxylic acid (DABCYL) conjugated to a B_2R antagonist, and (D) the BFQR mechanism based biosensor constructed on live HEK293 cells.

and in crude cell lysates.[25] This may provide a rational strategy for designing turn-on-type fluorescent biosensors. Similarly, Johnsson et al. have reported an hCA-based biosensor where displacement of an external ligand with the intramolecular ligand causes distance changes between the two fluorophores installed on the hCA scaffold.[34]

3.2. On Cell Application: Chemical Labeling of B_2R on the Surface of Live Cells. Given the high efficiency and selectivity of the multivalent AGD-based protein labeling approach, we attempted to label the bradykinin B_2 receptor (B_2R), a G-protein coupled receptor, on live cell surfaces.[18] We synthesized a multivalent AGD catalyst containing a B_2R-selective antagonist peptide as the ligand and three DMAP groups (Figure 12A). A cell-impermeable fluorescein (Fl) type acyl donor and human embryonic kidney (HEK) 293 cells transfected with B_2R harboring the plasmid were prepared as the experimental setup (Figure 12A). After incubation of the HEK293 cells with the AGD catalyst and acyl donor, the cells were extensively washed and observed by conforcal laser scanning microscopy (CLSM). A strong fluorescence was detected on the cell surfaces (Figure 12A). Kinetic analysis indicated ~85% of the entire cell population of B_2R was labeled within 30 min. To further examine the labeling reaction, the same labeling experiments were performed with a biotin (Bt)-type acyl donor (Figure 12A). Using an anti-B_2R antibody and a biotin-blotting analysis, we detected a single broad band that corresponded to the biotinylated B_2R (Figure 12B). Importantly, no significant change in cell morphology and intracellular Ca^{2+} flux induced by the agonist were observed, clearly demonstrating the applicability of the current approach for on-cell protein labeling.

A large number of fluorescent-based GPCR biosensors have been reported on live cells.[35] Two approaches have been mainly taken to study ligand binding of GPCRs. First, FRET between a fluorescent-labeled receptor and a fluorescently modified ligand has been used to directly monitor ligand binding.[36] Second, two fluorophores were inserted into one GPCR and the ligand-induced conformational change was detected by FRET.[37] The former gives a larger fluorescence change as the dynamic range is greater. We thus constructed a BFQR-based B_2R biosensor using a live cell system. Addition of the quencher-conjugated B_2R antagonist (Figure 12C) to the Fl-modified B_2R (Fl-B_2R) on HEK293 cells resulted in a reduction of fluorescence from the cell membranes (Figure 12D). Subsequent addition of a high-affinity antagonist, HOE 140, resulted in the recovery of the fluorescence intensity (Figure 12D). These fluorescence

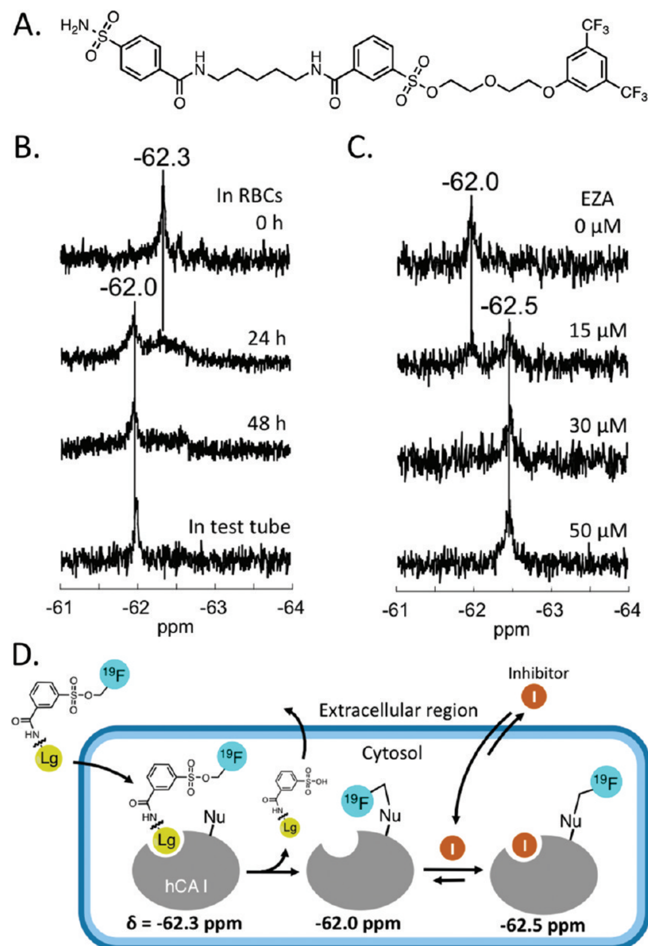

FIGURE 13. (A) Chemical structure of the ^{19}F-type LDT reagent, (B) monitoring of ^{19}F-labeling, and (C) the EZA binding processes by in cell NMR, and (D) schematic illustration of the in cell ^{19}F-NMR biosensor by LDT chemistry.

changes represent ~80% of the total fluorescence from Fl-B_2R, whereas only an ~10% change was reported for a CFP/FlAsH-tetracysteine system constructed on human adenosine A_{2A} receptors.[37] As a comparison, we also attempted to construct a BFQR-based biosensor using a conventional genetic technique, where we prepared an enhanced green fluorescence protein (EGFP) fused B_2R and expressed the EGFP-B_2R on HEK293 cells. In contrast to the Fl-B_2R system, no quenching of EGFP fluorescence was observed upon addition of the quencher, presumably due to the greater distance between EGFP and the quencher. These results established the advantage of our AGD-based chemical modification of proteins for biosensor construction on live cell surfaces.

3.3. In Cell Application: A ^{19}F-NMR-Based Biosensor in Living Cells. The chief advantage of our affinity labeling (or chemical labeling in general) is that diverse chemical probes can be readily chosen depending upon the detection modes.

^{19}F-NMR·MRI is expected to be a promising alternative to the conventional ^{1}H-MRI technique. ^{19}F has a high gyromagnetic ratio of 40.05 MHz/T (42.58 MHz/T for ^{1}H) and 100% natural abundance, yet there is no ^{19}F signal detectable in an animal body.[38] We thus designed a ^{19}F-type LDT reagent (Figure 13A) and have monitored the labeling process of endogenously expressed hCA in intact RBCs. Despite the complex cellular environment, the ^{19}F signal was clearly detected by in cell NMR. The original ^{19}F-peak at −62.3 ppm, which is identical to that of the ^{19}F-type LDT reagent bound to hCAI, was shifted to −62.0 ppm upon incubation at 25 °C for 48 h (Figure 13B). The chemical shift of the new peak was assigned to that of ^{19}F-labeled hCAI without the ligand, indicating that hCAI labeling occurs even in the miscellaneous interior of RBCs. We then incubated the labeled hCAI in RBCs with several CA inhibitors. Upon increase of the extracellular concentration of EZA, a new peak at −62.5 ppm appeared at the expense of the initial peak at −62.0 ppm (Figure 13C). This change was ascribed to the binding of EZA to ^{19}F-labeled hCAI in RBC, demonstrating that a ^{19}F-NMR based biosensor was successfully constructed in the cell from an endogenous protein using LDT chemistry (Figure 13D).

4. Concluding Remarks and Perspectives

Due to the paradigm shift of recent protein research, visualization and functional analysis of individual proteins in their native habitats has become highly desirable. Herein, we have summarized new chemical approaches to label and monitor the activity of individual proteins in their native environments. Compared with other protein labeling techniques, the approaches described above are unique in that (1) no preidentification of target proteins is required at the genetic level, (2) the target selectivity is achieved primarily by protein−ligand interactions, (3) the labeled proteins retain their native functions, and (4) endogenous proteins in living systems can be targeted and monitored. With the high selectivity and biocompatibility of the present techniques (especially with LDT and AGD), labeling of target proteins was achieved under miscellaneous native environments, that is, in or on cells and in living animals. Significantly, the present techniques enable the monitoring of important biological events such as ligand binding in living cells. Further, given the modular strategy, the selective module (ligand) and the read-out module (probe) can be creatively designed, providing a "molecular toolbox" for studying a wide variety of proteins with various biophysical techniques.

With the present techniques in hand, particularly important biological questions can be answered. Introduction of other functionalities, such as photo-cross-linkers or bioorthogonal reaction handles, may expand the applications to investigate biomolecular interactions, such as protein−protein, protein−DNA/RNA, and protein−lipid interactions, in living cells.[39] In addition, the chemistry-based approaches should allow for labeling of other important biological components, such as sugars[40] and lipids.[41]

One of the drawbacks of our current approach appears to be the inability to predict which amino acid residue will be labeled by each method. We have only shown that the amino acids proximal to the ligand binding pocket can be selectively modified. In this regard, genetic or metabolic incorporation of bioorthogonal groups exhibit more advantages. We are now empirically collecting data to describe amino acid selectivity by each method: tyrosine, histidine, glutamic acid and aspartic acid by LDT and tyrosine and lysine by AGD. Further, our recent reports suggest that fine-tuning of the chemical structures of the labeling agents,[39] as well as the use of a new reactive group,[42] can provide a gain in selectivity of the target amino acid. In combination with computational approaches, these experimental data may provide further insight in designing new labeling agents for selective labeling of native proteins *in vitro* as well as *in vivo*. We believe that such efforts may establish a new paradigm of synthetic chemistry in living cells/systems, which facilitates an understanding of complex biological systems and contributes to pharmaceuticals.

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FOOTNOTES

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