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Review

# Protein Chemical Modification on Endogenous Amino Acids

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Chemical modification of protein is an arduous but fruitful task. Many chemical methods have been developed for such purpose by carefully balancing reactivity and selectivity. Now both chemists and biologists have in hand an arsenal of tools from which they can select a relevant reaction to tackle their problems. This review focuses on the various chemical transformations available for selective modification of proteins. It also provides a brief overview of some of their main applications, including detection of protein interactions, preparation of bioconjugates, and protein microarrays.

## Introduction

Protein modification is of major interest in chemical biology. Indeed, many intra- and extracellular events depend on an occurrence of a specific chemical change that drives the change in both protein structure and function. These chemical modifications are commonly referred to as posttranslational modifications (PTMs), as they occur after the protein biosynthesis step, i.e., the translation. PTMs and proteins involved in mediating their incorporation into target proteins add additional layers to functional properties and diversities in the proteome and are the key to a number of crucial biological processes. For example, prior to degradation, proteins are often modified several times, leading to profound variation in their behavior. In general, incorporation of PTMs takes advantage of the chemical reactivity of the amino acid side chains and leads to specific functional outcomes. Upon phosphorylation (addition of a phosphate group to the side chain hydroxyl group of a serine, threonine, and tyrosine residue), conformational switches (de)activate protein-protein interactions and downstream events; upon farnesylation (addition of a farnesyl group to the side chain thio group of a cysteine residue), proteins get translocated to the membrane; and upon ubiquitination (addition of a small protein, ubiquitin, to the side chain amino group of a lysine residue), proteins are recruited by the 26S proteasome for degradation. Proteins can also undergo glycosylation, addition of a saccharide units; acetylation, addition of acetyl groups to the side chain amino group of a lysine residue; formylation, in which a formyl group is added to the N terminus of a protein; amidation, in which a formyl group is added at the protein C terminus; sumoylation, addition of a small protein, SUMO, to the side chain amino group of a lysine residue; and biotinylation, addition of a biotin molecule, to mention only a few. Overall, it's fair to say that selective modification of proteins is a key step in most biological processes. Nature uses covalent modification of proteins to modulate their function. As such, many natural products, originating from millions of years of evolution, have adapted their structure for covalent binding to proteins (Drahl et al., 2005; Pucheault, 2008).

Techniques that enable artificial chemical modification of proteins are therefore highly attractive, allowing for a more thorough understanding of molecular mechanisms, and many such methods have been developed over the last 20 years (Carrico, 2008; Francis, 2008; Sletten and Bertozzi, 2009). Some can be used directly in cells, while some have even been used in living animals. Although the goal may be similar, the means of attaining specific PTM artificially can differ significantly. An interesting approach from Peter Schultz allows for genetic encoding of unnatural amino acids (Deiters et al., 2003; Wang and Schultz, 2004). In this case, produced proteins display chemical functionalities that are not usually encountered within a cell, such as aryl iodides, boronic acids, or alkynes. Thus, proteins are further selectively modified in the second step using standard organometallic catalysis through various cross-coupling reactions (Dibowski and Schmidtchen, 1998). However, these methods require target pre-identification to enable genetic modification, limiting the scope of the method. Hence, this review will be limited to the chemical modification of proteins bearing solely naturally occurring amino acids. As a results, all commonly used techniques using proteins bearing unnatural functions such as alkynes or azides, including "Click Chemistry" reactions, will not be detailed hereafter and we refer those interested in learning more to a recently published review on the topic (Sletten and Bertozzi, 2009).

## Chemical Transformations Leading to Protein Covalent Modifications

Site-selective chemical modification of a protein requires two key features: an efficient reaction and an interesting molecule to attach. The molecule must relate to the desired application, while the reaction represents the means of attaining the modified protein in high yield and functional form. Performing chemical reactions on a biological macromolecule and maintaining its integrity is not an easy task. The primary reason relates to the distinct requirements for performing chemistry within a living organism as compared with standard organic synthesis. Below, we outline the key requirements for biocompatible chemical transformations that pose challenges to development of successful strategies.

### *Water Is the Sole Solvent*

Cells can tolerate a small quantity of alcohol-based solvent or DMSO, but the inclusion of these solvents induces high stress and significantly modifies normal cellular behavior.

### A Neutral pH Is Required

A pH between 6 and 8 should be maintained in order to minimize the effect on the processes of the cell.

### Ambient Temperature (Up to 40°C)

Cells can only survive within a narrow temperature range, requiring reactions to be performed near ambient temperature.

### Kinetics, which Adapted to the Observed Phenomenon (on the Hour Scale)

From the chemical standpoint, kinetics can deeply differ between room temperature (20°C) and 37°C, but few degrees are irrelevant.

### Low Reactant Concentrations

High concentration induces cell stress, leading to abnormal behavior. This means kinetic constants must be orders of magnitude higher than traditional organic chemistry where usual concentrations are in the molar range.

### Nontoxic Reagents

This factor is strongly related to concentration issues, as most molecules that display beneficial activity at low concentration can be toxic at higher levels.

These criteria should be seen more as guidelines than dogma, and several successful applications detailed below don't fully conform to them. Hence, most of these methods have so far only been applied in vitro to purified proteins. Indeed, a method that fulfills all conditions has a better chance not to interfere with normal protein function, even in vitro. Overall, most reactions used for protein modification use nucleophilic residues. So far, lysine, cysteine, and tyrosine residues have been widely studied. A few other examples use aspartic acid or histidine modification, but examples are scarce.

## Residue-Specific Strategies and Modifications

Lysines bear a primary amine moiety and although their side chains are protonated under physiological pH, they can still react as nucleophiles. N-hydroxysuccimide (NHS) esters, for example, react in the presence of an amine without exogenous reagents such as bases, which are usually required during peptide type couplings. In the presence of a primary amine (lysine), NHS esters irreversibly form the amide, and NHS is released in the medium (Figure 1A). Many NHS derivatives for the preparation of fluorescent probes and affinity reagents are now commercially available. Some sulfonated derivatives of NHS esters are appealing, as they allow better water solubility of the probe when used under physiological conditions. These NHS derivatives can all be used directly for in vitro labeling of a protein. Similarly, isothiocyanates react with primary amines, leading quantitatively to thiourea (Figure 1B). However, the optimal pH for an isothiocyanate reaction (9 to 9.5) on lysine is higher than for the formation of NHS esters (8 to 9) and may be unsuitable for modifying alkaline-sensitive proteins. Additionally, due to their high reactivity toward most nucleophiles, NHS esters and isothiocyanates are difficult to use directly in cells. As nucleophiles, lysine amino group can also irreversibly open 1H-benzo[d][1,3]oxazine-2,4-dione, leading to orthoaminebenzamide (Figure 1C). These modified proteins can be further functionalized using an oxidative strategy with $NaIO_4$ and a dialkyl acyl phenylenediamine (Figure 1D) (Hooker et al., 2006). Another approach relies on the reductive amination of an aldehyde. Namely, imine or Schiff base formation occurs rapidly and reversibly with both lysine and N terminus. In a second step, the imine is irreversibly reduced into the secondary amine using water-compatible hydrides such as $NaBH_3CN$ (Figure 1E) (Jentoft and Dearborn, 1979). This can allow, for example, the formation of glycoconjugates of bovine serum albumin (Gildersleeve et al., 2008). Alternatively, overcoming the reversibility of the imine formation, a 6π-aza electrocyclization (Figure 1F) (Tanaka et al., 1999) will lead, after ester hydrolysis, to the irreversible formation of the corresponding zwitterions (Figure 1G) (Tanaka et al., 2008b).

For arginine residues, a pyrimidine derivative can be formed using two of the three nitrogens of the guanine in the ring (Figure 1H) (Oya et al., 1999). This methodology proceeds at a 100 mM concentration at 37°C in a phosphate buffer (pH 7.4). The reaction was first developed on a simple model, $N^{\alpha}$-Acetyl-L-Arginine, then applied to bovine serum albumin to prove the selective modification of arginine. While efficient, this method is limited by its reaction time (14 days), which makes it inapplicable in cells.

Epoxides have often been used to modify proteins and are subsequently reacted with various nucleophiles, leading to random protein surface modification. However, in some cases it has been shown that selective histidine modification occurs (Figure 1I) (Chen et al., 2003) in a way similar to what is observed with epoxide-derived natural products such as fumagillin or epoxomycin.

Another approach relies on the selective modification of carboxylic groups present on glutamate, aspartate, or the C terminus of a protein. This method consists of standard peptidic coupling reactions creating amide bonds with this terminal functionality and using a water-soluble carbodiimide such as *N*-ethyl-3-*N'*,*N'*-dimethylaminopropylcarbodiimide (EDC). This reagent is not stable under all pH conditions (Gilles et al., 1990), but has been used to specifically modify glutamate residues on the protein coat of the tobacco mosaic virus (Schlick et al., 2005). This modification creates an amide bond between this residue and exogenous amines in phosphate or HEPES buffer (pH 7.4) at room temperature (Figure 1J). However, the use of EDC results in the formation of a byproduct (*N*-acyl urea) originating from the rearrangement of the *O*-acylisouronium intermediate. To suppress this pathway and decrease the formation of this side product the authors efficiently used an excess of hydroxybenzotriazole (HOBt).

In addition to nitrogen-containing residues, the phenol groups present on tyrosine can also react as nucleophiles through addition to multiple activated bonds. The first method relies on the addition of the phenol to a diazonium salt. Originally, this reaction was developed for the synthesis of azo compounds, a central motif in the dye industry. In that instance, the substitution selectively occurred ortho to the phenol group (Figure 2K) (Hooker et al., 2004). However, the substrate scope was initially limited to aryl diazonium salts bearing electron-withdrawing groups in the para position. Interestingly, the azo bond can be further reduced into the aniline and transformed into a stable benzoxazine with a substituted acrylamide (Figure 2L). Overall, 180 tyrosine residues from the interior surface of a MS2 bacteriophage were covalently and specifically modified using this method (Hooker et al., 2004). Alternatively, when a ketone-derived aryldiazonium is used, further alkoxy amine condensation leads to the formation of stable oxime ethers (Figure 2M). This approach

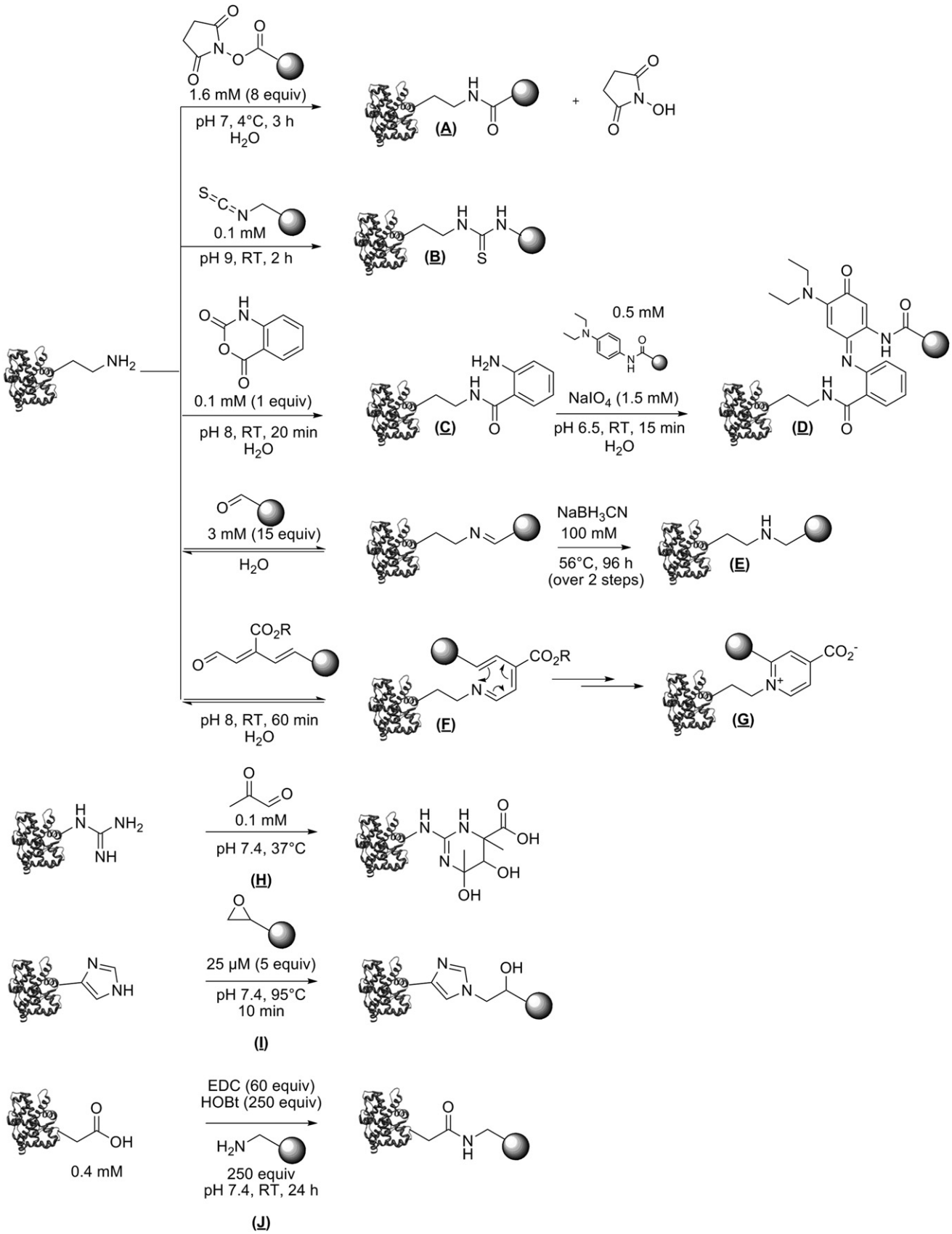


**Figure 1. Reactivity of Lysine, Arginine, Histidine, Aspartic Acid, Glutamic Acid, and C Termini**

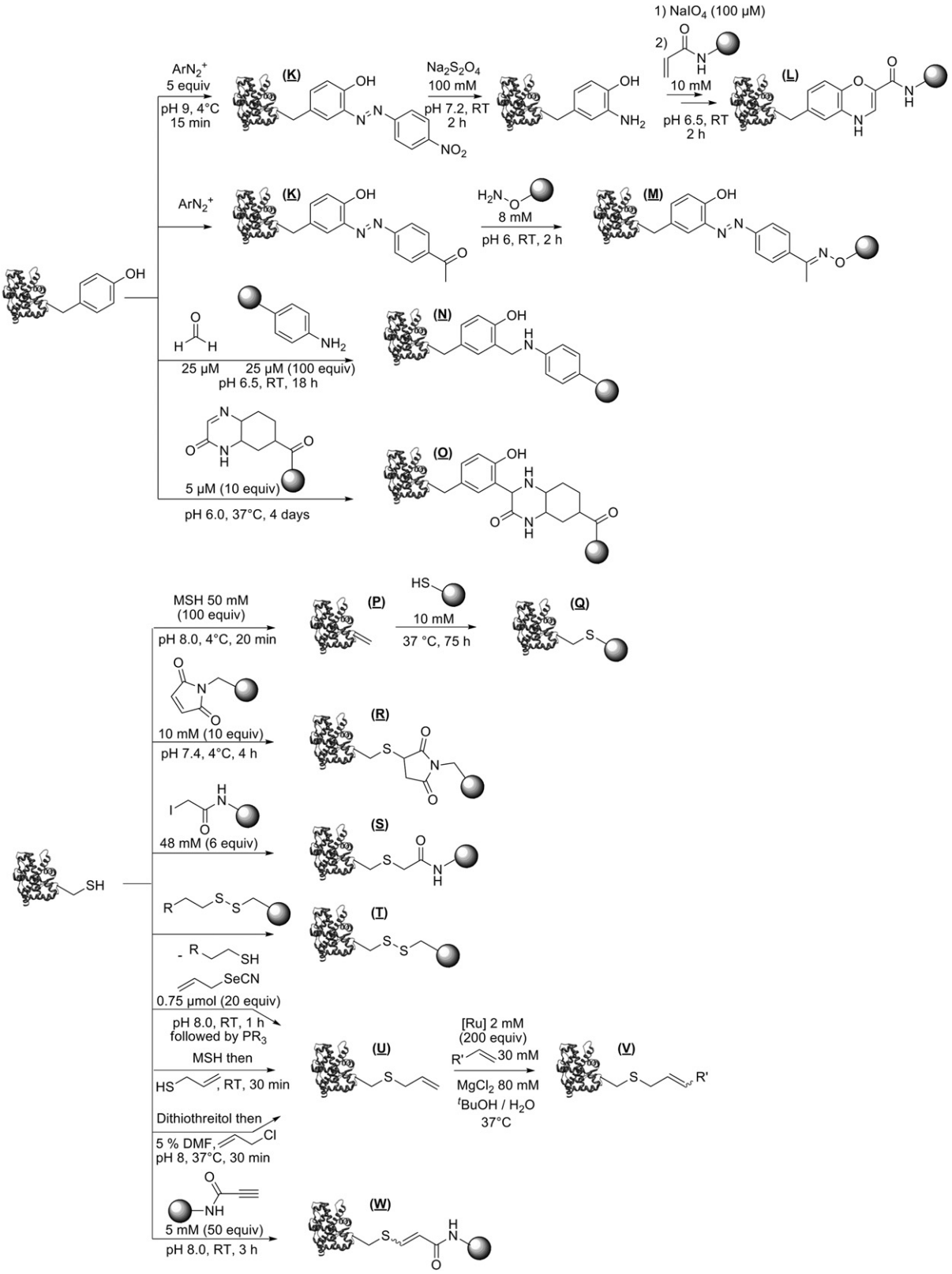


**Figure 2. Reactivity of Cysteine and Tyrosine**

 

was used for the modification of the external surface of the tobacco mosaic virus (Schlick et al., 2005). Another approach relies on using a Mannich three-component reaction. This methodology, which requires an aldehyde and an amine as additional reactants, allows for tyrosine residues to be modified with a high selectivity (Figure 1N) (Joshi et al., 2004). The reaction necessitates only mild conditions and low millimolar concentration of reagents at pH 5.5–6.5. This reaction was successfully applied to proteins displaying tyrosine on their surface (lysozyme, RNase A, and chymotrypsinogen A), but failed for horse heart myoglobin, which has tyrosine residues deeply buried in the three dimensional structure, proving the high selectivity of the method. However, even if the contribution of tryptophan in the Mannich reaction is considered, it doesn't prevent the selective modification of tyrosine residue (McFarland et al., 2008). Recently, preformed imines were shown to react equally well in the fluorescent labeling of proteins (Figure 2O) (Guo et al., 2009).

Cysteine residues can be modified using the thiol side chain. The thiol moiety is more nucleophilic than a primary amine, especially at a pH below 9, where the amine is protonated. Consequently, cysteine often reacts faster than lysine, resulting in a selective modification of cysteine over lysine residues. However, free thiols are relatively rare in proteins and they are often found as an oxidized dimer, known as a disulfide form. Therefore, it is often required to expose proteins to reducing agents, such as dithiothreitol, as a preemptive treatment to free the thiol groups. Oxidative elimination of cysteine upon treatment with O-mesitylenesulfonylhydroxylamine (MSH) under basic conditions affords dehydroalanine (Figure 2P) (Bernardes et al., 2008). These modified proteins are subsequently modified by conjugate addition of thiols (Chalker et al., 2009a) leading to thioether analogs (Figure 2Q). Maleimides react selectively and stoichiometrically with thiols (Figure 2R) (Kim et al., 2008). These scaffolds are stable under biological conditions and are widely used to selectively modify cysteines, as lysine often fails to react under the same reaction conditions. Alternatively, an alkylation can be performed using iodoacetamide (Figure 2S). These iodoalkanes are more electrophilic than regular alkyl iodides due to the presence of the carbonyl group. The resulting reaction is analogous to the formation of a thioether. In some specific cases, these reactive species can even react with methionine residues (Gundlach et al., 1959). Surprisingly, though one could imagine performing a direct allylation of a protein using an allyl halide to form the allylic thioether, this method had only been applied extensively to the modification of peptides and was described only recently with regard to a protein (Chalker et al., 2009b). S-Allyl cysteine can also be generated by using a one-pot treatment with MSH followed by 2-propen-1-thiol. These allyl sulfides can be further functionalized using olefin metathesis catalysts, thus allowing glycosylation or PEGylation on protein surface through cross-metathesis (Figure 2V) (Lin et al., 2009). Thiols can also be added to electron-poor triple bonds such as alkynoic amides (Figure 2W). The resulting thioolefin can be isolated as such but can also undergo a second addition resulting into protein release (Shiu et al., 2009). The thiol side chain on cysteine can also be used in other reactions. Indeed, thiols are known to form disulfide bonds under oxidative conditions. This reaction is similar to the one involved in the cysteine-cystine equilibrium that helps direct protein tertiary structure. Therefore, upon addition of a disulfide probe, an equilibrium is established leading to the covalent attachment of the probe through a disulfide bond (Figure 2T) (Rademann, 2004). This reaction is selective for cysteine residues and very mild, but the link is not resistant to the addition of mercaptoethanol or dithiothreitol. In case a stronger linkage is required, Crich and co-workers adapted the chemistry developed by Sharpless and Baldwin involving the rearrangement and reduction of allylic disulfides and allylic diselenides for use with proteins (Figure 2U) (Chalker et al., 2009b; Crich et al., 2006, 2007).

## Organometallic Catalysis-Based Covalent Modification

All the previously described methods rely on the use of highly reactive reagents to allow the modification of a nitrogen- or a sulfur-based nucleophile. As such, these reagents have a potential to suffer from poor selectivity when used in cellulo instead of in vitro on purified proteins. To overcome this obstacle, transition metal complexes have elegantly been used by Antos and Francis (2006) to achieve selective labeling of proteins (van Maarseveen et al., 2006). Albeit not yet applied to cells, the approach uses the modularity of transition metal complexes, leading to exceptional activity and selectivity while maintaining significant aqueous solubility. Owing to the catalytic nature of the reagent, low loading of the transition metal complex is required to achieve the same level of labeling.

In the case of reductive amination, a highly reactive reducing agent such as $NaBH_4$ or $NaBH_3CN$ (Figure 1E) could be replaced by the less harmful sodium formate. This reducing agent fails to react alone on an imine, thus the system requires the addition of a transition metal catalyst such as an iridium complex (Figure 3A). The method is based on a classical method for reduction of an imine through a hydride transfer type mechanism (Ogo et al., 2004). For proteins, 25 mM $HCO_2Na$ in phosphate buffer (pH 7.4) at room temperature can provide selective and irreversible labeling of lysine and N terminus, even at low concentrations (10 μM) (McFarland and Francis, 2005).

Similarly, for allylation of nucleophilic residues, standard allylic electrophiles, such as allyl bromide, were either non-selective or failed to react. In the presence of a water-soluble complex of palladium, the Tsuji-Trost reaction between tyrosine and allylic acetates led to the covalent substitution product (Figure 3B). This reaction proceeds through a π-allyl palladium complex generated by oxidative addition of the palladium onto the carbon-acetate bond. This key step enhances the electrophilicity of the allylic moiety, enabling reaction with weaker nucleophiles such as phenolate. This reaction was successfully applied to selective chymotrypsinogen A tyrosine labeling at a low concentration (5 μM) (Tilley and Francis, 2006).

This approach is not limited to improving stoichiometric labeling. It can also provide a new transformation for selective bioconjugation to residues that are difficult to modify with other methods. For example, tryptophan is irreversibly modified by diazoacetate derivatives in the presence of Rh(II) complexes (Figure 3C). Diazoesters alone fail to react, but the addition of a rhodium catalyst decomposes the diazoester to a metallocarbenoid that performs an easy C-H insertion (Antos and Francis, 2004). Initially, the reaction was limited by the requirement of acidic conditions (pH < 3.5) and by the use of $H_2NOH.HCl$ as an additive. Recently, however, by optimizing the nature of the

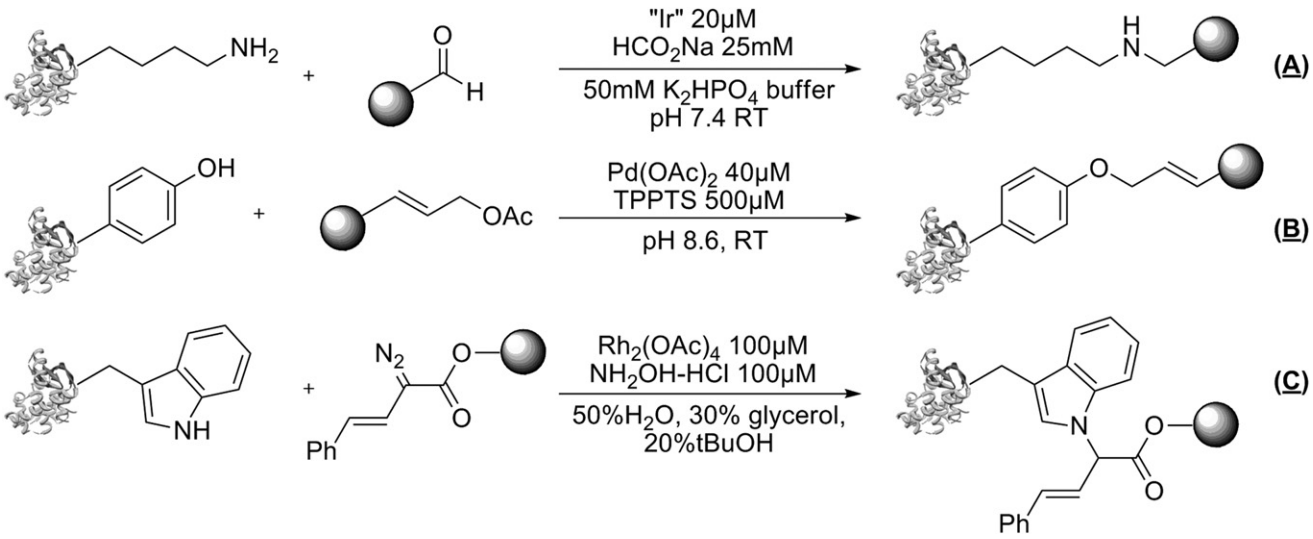


Figure 3. Transition Metal-Catalyzed Bioconjugation

additive, this reaction has been shown to be efficient at pH 6, which is suitable for most biological applications (Antos et al., 2009).

## Photochemically Induced Bioconjugation

As an alternative, precise wavelengths of light can be used to generate highly reactive species, such as carbenes, nitrenes, and radicals originating from diazirine, arylazide, and benzophenone decomposition, respectively (Brunner, 1993; Knowles, 1972; Singh et al., 1962; Tanaka et al., 2008a). Owing to their extreme reactivity, these species react rapidly with any molecule, regardless of its nature. This means that they are, on one hand, likely to get hydrolyzed by the solvent and, on the other, react with any molecule of interest, including DNA, oligosaccharides, or proteins. As photoactivation is largely independent of other parameters, this method can be used in combination with other molecular biology techniques. However, labeling is often neither site nor residue selective.

The benzophenone groups are usually chemically stable, but can still be activated under UV irradiation at 350–360 nm to give the corresponding benzhydril biradical (Figure 4A). This activation is reversible, as the diradical transforms back to the benzophenone after 120 μs. Activated benzophenone usually reacts fast with all neighboring C-H bonds. In the case of hydrolysis, the ketal evolves back to the ketone. Overall, benzophenone has a tendency to react preferentially with methionine residues, but the selectivity is influenced significantly by both electronic effects and the steric hindrance created by the aromatic groups.

Under short wavelength photoactivation (usually around 254 nm), arylazides extrude nitrogen and generate a short-lived nitrene species (Figure 4B). Similar to the carbene, they react with any C-H or heteroatom-H bond to create an insertion covalent adduct. However, if no efficient labeling occurs, nitrenes rearrange into ketenimines. These compounds are less reactive, but significantly more stable, and react with all nucleophiles leading to non-specific labeling. Unfortunately, both photo-crosslinkers can cause cell damage and even lead to cell death. In addition, as mentioned, the nitrenes can rearrange to the ketimines, leading to formation of undesired side products.

Diazirines are photoactivated with light over 300 nm, which, like the benzophenones, makes them less prone to generating photodamage to biological samples (Hashimoto and Hatanaka, 2008). Upon activation, diazirines convert to carbenes following nitrogen loss. Carbenes insert rapidly ($t_{1/2}$ in the nanosecond range) into any atom-hydrogen bond, leading to random adducts. Two main diazirines are commonly used, trifluoromethylaryl diazirines (Figure 4C) and methylalkyldiazirines (Figure 4D). Diazirines can rearrange into the diazo compound, which, in the case of alkyl diazirine, forms an unstable intermediate that reacts rapidly with nucleophiles, including water. However, the small size of alkyldiazirines allows access to reaction sites that are unattainable by larger diazirines. Trifluoromethylaryl diazo isomers are significantly more stable, leading to an unproductive steady state and reducing the efficiency of the labeling process, but increasing specificity.

## Protein Crosslinking

The oxidative crosslinking methodology couples two adjacent residues such as cysteine, tryptophan, or tyrosine (Fancy et al., 2000). Using this technique, two proteins can be linked together, thus providing a chemical analog to fusion proteins. This allows detection of protein-protein interactions with a direct readout, providing one of the proteins is known. In the case of tyrosine, the key step in the process is the oxidation of the phenol that generates a highly reactive tyrosyl radical (Figure 5A). This radical is subsequently attacked by any nucleophile present in the spatial vicinity, such as a cysteine or a tyrosine from another protein. This oxidation can be induced by various complexes, including Gly-Gly-His Nickel(II) complex (GGH-Ni(II)) (Brown et al., 1995) and Pd(II)-porphyrin (Kim et al., 1999). In the first example, following activation by magnesium monoperoxyphtalate or oxone, the active species for this reaction is believed to be a Ni(III) oxo intermediate. With palladium complexes, the activation is induced photochemically, owing to the porphyrin

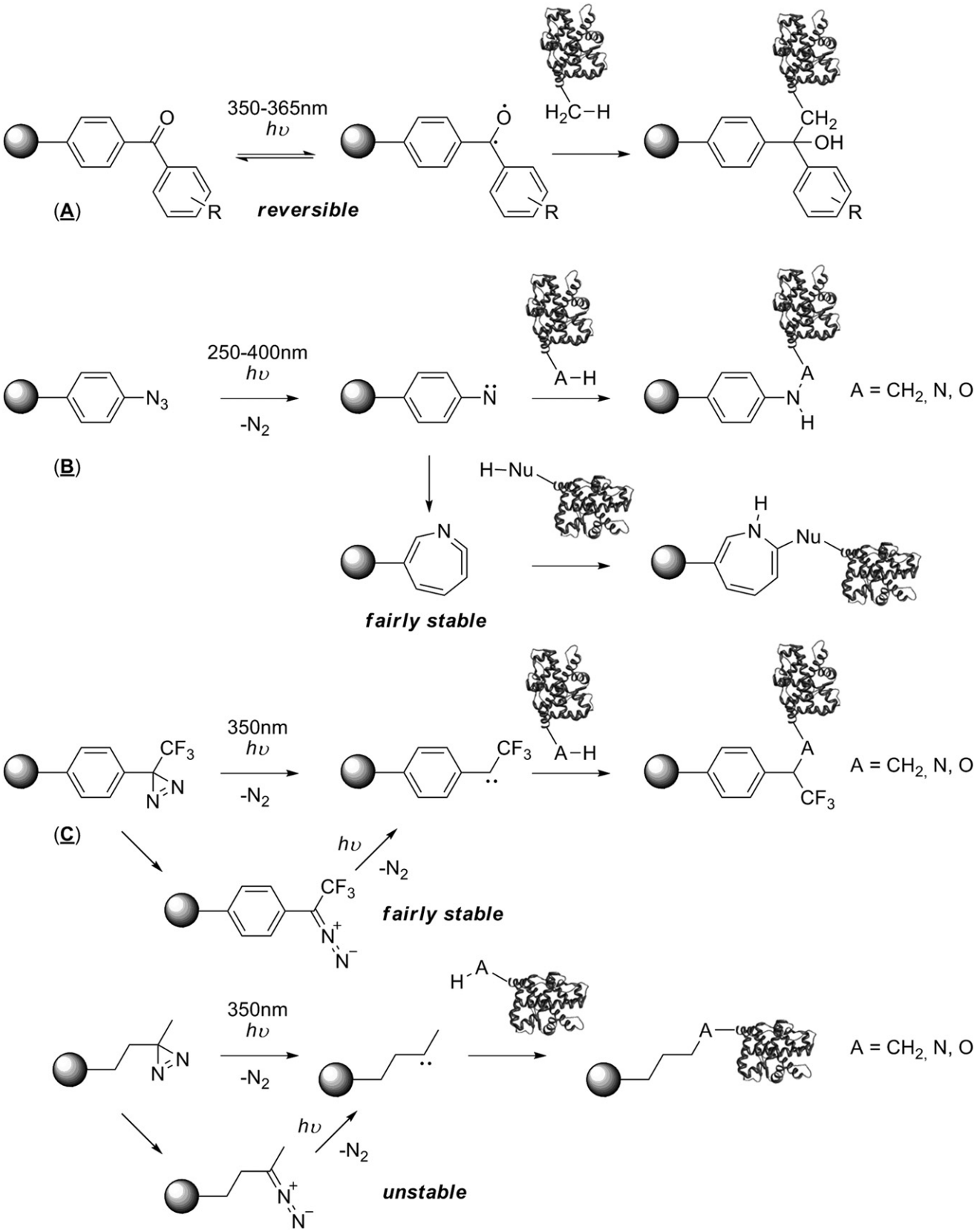


Figure 4. Protein Photolabeling

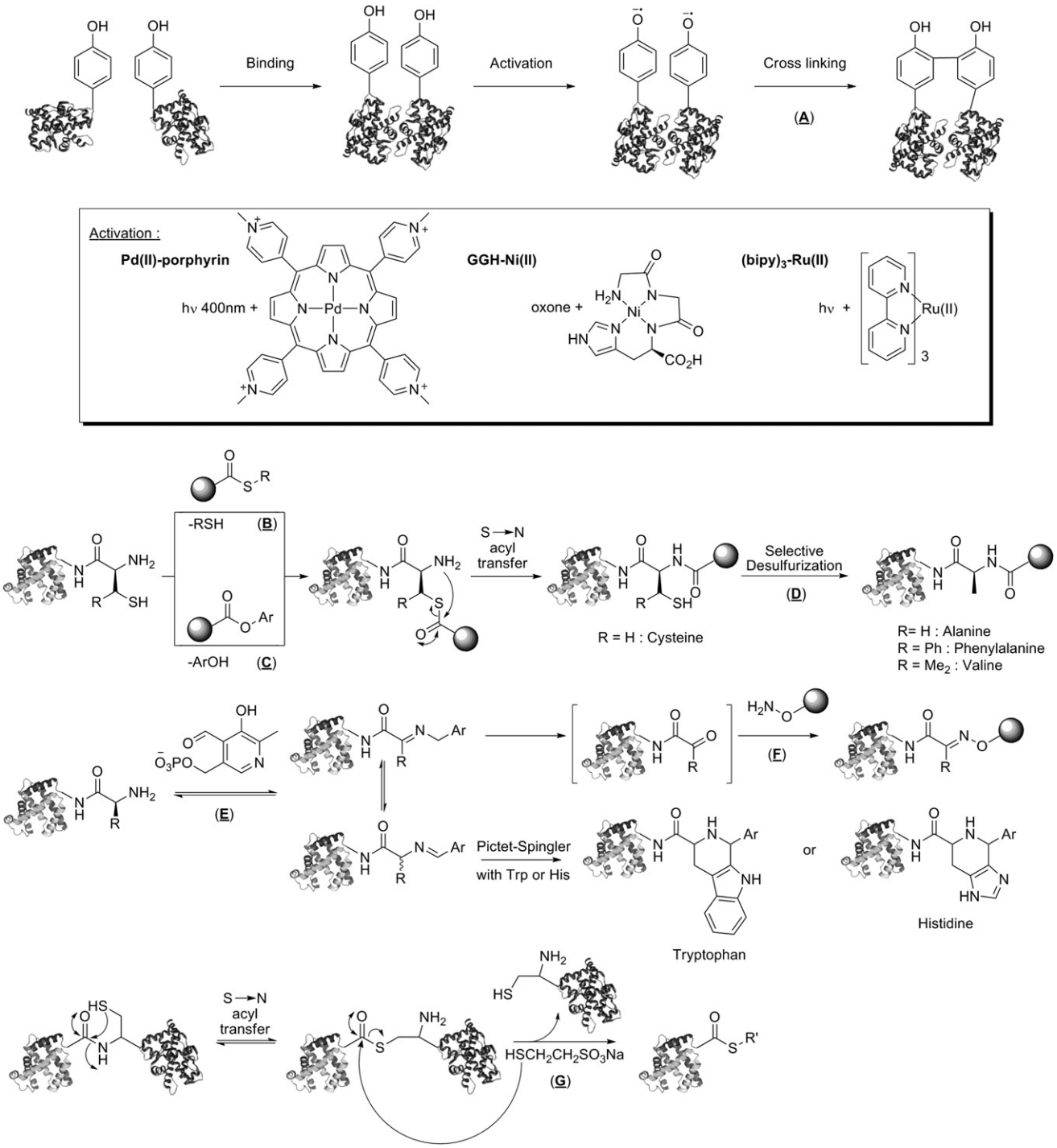


Figure 5. Oxidative Coupling and N Terminus Modification

chromophore. Alternatively, Ru(II)-bipy$_3^{2+}$ can induce faster reactions than chemically induced oxidative crosslinking (Meunier et al., 2004). This method has been used for crosslinking an azide-derived thiol on cowpea mosaic virus capsid protein (Meunier et al., 2004). Specifically, the azido propylamide derived from cystine was reacted with a large excess of GGH-Ni(II) complex and chemical initiator. The resulting azido-functionalized proteins were subsequently modified with a fluorescent label bearing an alkyne using click chemistry.

## Specific Modification of N and C Termini

The primary method used for modification of N termini is called native chemical ligation. It relies on the rearrangement of an amino thioester into a thioamide. The application of this method to

a protein was first described by Dawson et al. (1994). In the case of endogenous proteins, this method is only applicable to protein with an N-terminal cysteine. Specifically, the thiol side chain reacts with a thioester, leading to an amino-substituted thioester, which can then undergo a S to N acyl transfer generating an amide (Figure 5B) (Hackenberger and Schwarzer, 2008; Yeo et al., 2004). This reaction can be followed by desulfurization (Figure 5D) or oxidation of the thiol, leading to alanine or dehydroalanine, respectively. Alternatively, oxoesters can be used instead of thioesters (Figure 5C). The general S to N migration principle remains identical, but relies on the low stability of phenolic esters instead of a slow transthioesterification. This method can be used for protein semisynthesis (Kent, 2009), even complex ones such as chemically engineered ion channels (Bayley et al., 2009), keeping its folding and its activity (Li et al., 2005).

A method for the specific modification of a protein's N terminus was reported by Gilmore et al. (2006) using transamination in the presence of pyridoxal-5′-phosphate (PLP). The PLP is a cofactor involved in various natural protein modifications such as elimination, decarboxylation, and transamination. Theoretically, all amino groups can undergo such transformation. However, in the absence of an enzyme, increased basicity of the proton in the position $\alpha$ to the N terminus greatly enhances the reaction rates under biological conditions. At the molecular level, the condensation of the N terminus with PLP at 37°C in phosphate buffer (pH 6.5) forms an intermediate imine, which is subsequently hydrolyzed into a ketone or an aldehyde (Figure 5E). This carbonyl group is then condensed with an alkoxyamine, generating an oxime ether that is stable toward hydrolysis (Figure 5F). This reaction was developed using horse heart myoglobin (which bears an N-terminal glycine residue) at low concentrations: 10 mM for PLP and 25 mM for phosphate buffer (Gilmore et al., 2006). Following careful optimization of the reaction conditions and substrate scope it was clear that the reaction works better with a protein bearing an N-terminal alanine, glycine, aspartic acid, glutamic acid, or aspargagine. The other N termini reacted as expected, with the exception of tryptophan and histidine, for which the Pictet-Spengler adducts were obtained in high yields (Scheck et al., 2008) and only lysine and glutamine failed to react. This interesting method was then applied successfully to the N-terminal selective modification of antibodies (Scheck and Francis, 2007).

Surprisingly, only a few methods are available in the literature for specific modification of C termini. However, the preparation of C-terminal thioesters required for N-terminal ligation (see above) is well documented and relies most of the time on a modified version of solid phase peptide synthesis methods (Hackenberger and Schwarzer, 2008). Alternatively, modification of C termini can be achieved via protein splicing at cysteine residues using intein-based methods (Flavell and Muir, 2009; Zhang et al., 2009) or through S to N acyl transfer followed by intermolecular thiol-thioester exchange with 2-mercaptoethanesulfonate (Figure 5G). The generated thioester can then be used to perform native chemical ligation followed by desulfurization at phenylalanine or valine (Figure 5B).

## Applications

Selective modifications of endogenous proteins remain a challenge, but the outcomes are worthwhile. Despite the intrinsic difficulties, protein modifications have found many useful applications. A molecule that is covalently attached to a protein can be designed to display various functionalities, which can be used for detection, purification, or modulating the function of the protein itself.

Several commonly used protein detection techniques are based on the use of covalently attached chromophores that allow monitoring of a fluorescent signaling and thus provide insight into the protein localization within cells or protein identification using SDS-PAGE. Numerous other strategies and epitopes can be used and specifically tailored to alternative detection techniques. Gadolinium complexes provide MRI contrast; spin labels can be detected using EPR; radioactive labels can also be used.

### Protein PEGylation

PEGylation of a protein provides significant modification of its solubility and bioavailability within an organism. Moreover, the presence of polymeric chains prevents interactions with degradation enzymes, increasing the lifetime of a protein and turning it into an attractive drug candidate (Veronese and Pasut, 2005). Methods for controlling the level of functionalization of a protein are highly desirable, as the stability in physiological media increases with the number of linked chains and the biological activity decreases. For example, a site-specific pegylated interferon $\alpha$ protein therapeutic was generated via an attachment of a maleimide-PEG to a recombinant cysteine-containing interferon $\alpha$, resulting in a PEGylated product with an enhanced circulating half-life and antitumor/antiviral activities (Bell et al., 2008). PEG-phenyl-isothiocyanate is highly stable against hydrolysis and can react with human serum albumin to increase its hydrodynamic volume and therefore might be used as a plasma expander (Meng et al., 2008). Maleimide-activated PEG can be used to obtain a stable specific cysteine mono-bioconjugation on a protein (Salmaso et al., 2009). For proteins featuring a disulfide bridge, a PEGylation agent containing an $\alpha,\beta$-unsaturated double bond and an $\alpha,\beta$'-sulfonyl group that is prone to elimination as sulfinic acid promotes the formation of a three-carbone bridge between the two-cysteine sulfur atoms (Lewis et al., 2008). *N*-succinimidyl carbamates such as PEG-$\beta$Ala-NHCO-OSu, derived from $\beta$-Alanine, display lower reactivity (compared to common PEGylation agents), providing increased selectivity toward the most nucleophilic amines (Pasut et al., 2008).

### Small Molecule Target Identification

One of the main uses of protein modification is the identification of the protein targets of small molecules. Indeed, appending a protein-reactive entity to a natural product can lead to a probe that will interact preferentially with a protein or a group of proteins based on the binding preference of the natural product. This allows subsequent use of classical detection methods, such as blotting or immunoprecipitation, for direct identification of potential protein targets. For this application the most widely used scaffolds are photoreactive entities, as they form a covalent bond between the protein and the small molecule regardless of the binding site. For example, this type of crosslinker was used to show direct binding between cyclopamine and the heptahelical bundle of Smoothened (Smo) (Chen et al., 2002). This validated Smo as a relevant therapeutic target in the treatment of

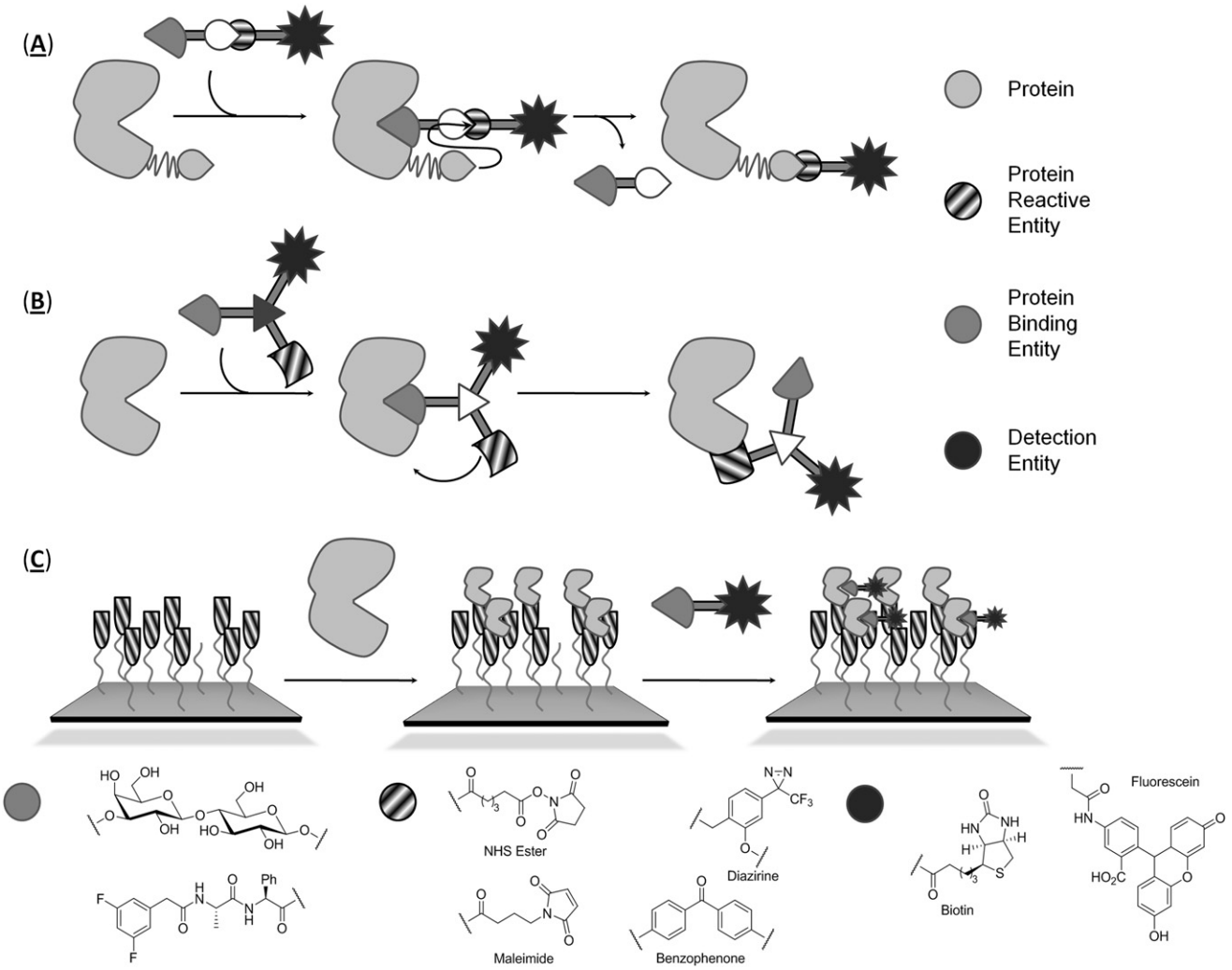


**Figure 6. Main Applications of Protein Covalent Modification**

Hedgehog-related diseases. A photoaffinity test was performed in phenol red-free DMEM at 37°C with 1 μCi of $^{125}$I-labeled azide-derived cyclopamine, photoactivated by irradiation at 254 nm. Confirmation was made using BODIPY-derived cyclopamine, providing protein localization in cells. This example emphasizes the importance of using complementary labeling/detection methods to afford the relative orientation of the puzzle pieces. Alternatively, selective mutation on a putative binding site residue by cysteine, combined with the use of iodoacetamide- or maleimide-derived small molecules, can provide confirmation of both the nature of the target protein and the location of the binding site. (Figure 6A).

## Trifunctional Probes

One elegant method used for the identification of the protein target of a small molecule relies on scaffolds bearing three distinct parts. The first part is the small molecule itself, which provides the general protein-ligand recognition. The second part provides for detection and can include fluorescence or a tag for affinity purification. The third part is the protein reactive motif, which generates a covalent protein ligand bond (Figure 6B).

Diazirine-based trifunctional probes represent one of the most popular choices with regard to probe design (Tomohiro et al., 2005). With this motif, a high wavelength photoactivation is sufficient for generating the reactive carbene. As such, the creation of a covalent bond between the scaffold and the protein is essentially harmless for the biological system. Such probes, combined with the use of solid phase synthesis, have allowed the direct identification of the binding site of DAPT, a potent inhibitor of γ-secretase (Kan et al., 2007). Several photoaffinity-based probes, derived from the ligand combined with biotin, have been evaluated toward Aβ40 generation. After controlling for their bioactivity, labeling experiments followed by purification and mass spectrometry analyses showed no real differences between diazirine- and benzophenone-based probes.

Indeed, due to their simplicity, benzophenone reactive groups have been widely used as photoactivable agents, showing the broad applicability of the method. SecinH3, a small molecule

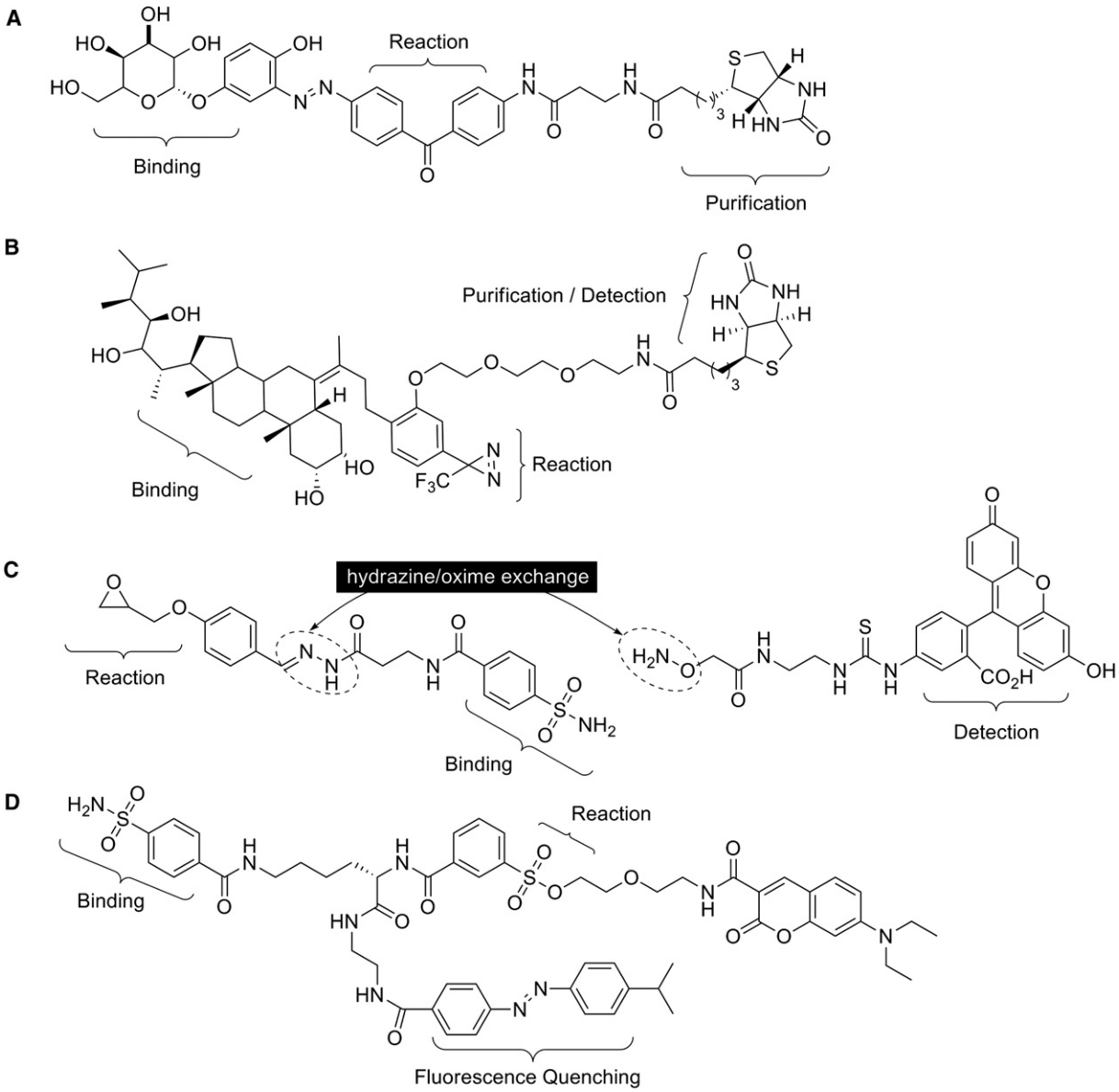


**Figure 7. Examples of Trifunctional Probes**
(A) Binding: arbutin; reaction: benzophenone biradical insertion; detection: purification with biotin (Qvit et al., 2008).
(B) Binding: brassinosteroid/BRII; reaction: carbene insertion with diazirine; detection: purification with biotin.
(C) Binding: benzensulfonamide/CAII; reaction: histidine epoxide opening; secondary detection by hydrazine-derived ligand/oxime-derived fluorescein exchange (Takaoka et al., 2006).
(D) Binding: benzensulfonamide/CAII; reaction: SN2 on tosylate; detection: fluorescence with 7-diethylaminocoumarin (Tsukiji et al., 2009b).

derived from medicinal screening, is known to be an inhibitor of cytohesins such as GEF and GTPases. Using a trifunctional probe containing benzophenone and biotin, SecinH3 was shown to bind directly to the sec7 domain of members of this protein family (Bi et al., 2008). This methodology has also been shown to work with carbohydrate derivatives such as arbutin, the natural ligand of β-glucoside transporter systems. Disubstituted benzophenones, with arbutin on one end and the label on the other (Figure 7A), were prepared in order to investigate translocation in mammalian cells, where no analog of the microbial transport system is known (Qvit et al., 2008).

As a proof of concept, β-galactoside containing probes have been prepared to show direct binding to galectin-1 and galectin-3 (Ballell et al., 2005). In this approach, the detection is not done directly with the protein ligand adduct. Instead, the probe is designed to enable further functionalization through [3+2] cycloaddition with a terminal azide and a fluorescent alkyne. Similarly, a Staudinger ligation with the same terminal azide can be used as a good alternative to the Huisgen-Sharpless click chemistry method. In the last example, a benzophenone-derived potassium isolespedezate was used to study the circadian rythmic leaf closing mechanism in leguminous plants (Fujii et al.,

2008). The mechanism of action of such probes is threefold. The first step is the binding of the ligand to its target protein, followed by reaction of the molecule with the protein. At this point, denaturing conditions can be used without breaking the probe-protein linkage. This is followed by derivatization of the probe with a moiety that will provide an easier readout. It can be a fluorescent scaffold for gel identification or a biotin derivative for further purification and analysis. This method is even more general than the previous one, as interference with the protein-ligand interaction is minimized. This has been applied using a histidine-driven epoxide opening on a bifunctional molecule (Takaoka et al., 2006), followed by hydrazine/oxime exchange replacing the ligand with a fluorescent scaffold (Figure 7C).

Finally, though most methods use orthogonal activable photoreactive moieties, several other examples can be found using simpler reactive groups in order to form a covalent bond with cysteine (Chen et al., 2003). Several reactive groups have been systematically evaluated, including trifluoromethyldiazirine, Michael acceptors, and benzyl halides. Epoxides turned out to be both efficient and selective in the presence of other proteins. Recently, a novel strategy with high target specificity was developed based on ligand-directed tosylate (LDT) chemistry (Tsukiji et al., 2009a). This method allows the site-specific introduction of synthetic probes to the surface of proteins with concomitant release of the affinity ligand. The new quenched LDT-mediated labeling (Q-LDT) directly converts a natural protein to a fluorescently labeled protein. The molecule bears four parts: the ligand, the reactive group, a fluorescent probe, and a fluorescence quencher. Upon labeling, the ligand-quencher fragment is released and only the fluorescent component is attached to the protein, turning on the fluorescence (Figure 7D) (Tsukiji et al., 2009b). This Q-LDT strategy has been successfully applied to carbonic anhydrase II (CAII) and has also been used to generate turn-on fluorescent biosensors toward CAII inhibitors.

## Protein Microarrays

With an arsenal of methodologies for protein modifications, immobilization of a protein on a surface (Figure 6C) is another major application (Griffiths, 2007). Protein microarrays serve two main purposes: finding molecules to which a protein binds or molecules with which it reacts (Lee and Mrksich, 2002). For binding, quantitative arrays are designed to measure the level of expression of a protein in a tissue: a lysate is applied on the protein array, and subsequently detected with fluorescent antibodies. The crude lysate can also be directly grafted onto an array, bypassing the prefunctionalization step in the so-called reverse phase array, while the detection remains identical. To study activity, a protein array is screened against fluorescently labeled small molecules or proteins. For example, a multitude of potential enzyme inhibitors can be evaluated when coapplied to the chip with a fluorogenic enzyme substrate. Almost all previously mentioned methods have been applied to this specific problem of how to immobilize covalently a protein on a surface without affecting its normal behavior. Two strategies are therefore possible depending on where the critical step stands. If the important parameter is controlling the protein linkage site, a relevant approach would probably be to modify the protein selectively and then attach it to the surface. On the contrary, if the relevant link site is not important or can't be predicted, one would probably prepare a surface bearing functions, which would react with all protein side chains, most often photoactivable entities. As such, protein are randomly attached to the surface, and at least a small portion of them would retain their activity. These specific applications have recently been reviewed (Jonkheijm et al., 2008; Wong et al., 2009).

## Bioconjugation

The synthesis of bioconjugates is critical to the study of the influence of posttranslational modifications of proteins. To this end, proteins can be functionalized artificially with oligosaccarides or oligonucleotides. Site-selective glycosylation of bovine hemoglobin (Hb) was achieved by conjugating a lactose derivative to Cys β93 of Hb, thus leading to a potential Hb-based oxygen carrier (Zhang et al., 2008). Carbohydrate-protein conjugates are used in cancer vaccines and bacterial vaccines and can be synthesized via reductive amination using sodium cyanoborohydride reagent (Gildersleeve et al., 2008; Wang et al., 2008). Peptides can be specifically attached to proteins via their N or C termini through tyrosine bioconjugation using a three-component Mannich-type strategy (Romanini and Francis, 2008). Conjugation with peptides enhances protection against enzymatic hydrolysis (Tugyi et al., 2008). Single-step and site-specific conjugation of bioactive peptides to proteins can be achieved via a self-contained succinimidyl bis-arylhydrazone, allowing formation of bioconjugates used for vaccines or mechanistic studies (Phillips et al., 2009). The bioconjugation can be inter- or intramolecular and used in combination with advanced mass spectrometry to map protein-protein interactions (Sinz, 2007a) or unravel drug-protein interactions (Muller et al., 2009; Sinz, 2007b, 2007c).

## Other Applications

One can imagine many other possible applications for native protein covalent modifications. For example, diazirine-derived DNA could unravel new DNA-protein interactions. This strategy was used (Shigdel et al., 2008) to study protein-DNA interactions in 10 mM Tris-HCl. After incubation, the samples were irradiated with 300 nm light for 10 min to allow for a high level of labeling. Owing to the relatively small size of the diazirine, this method may be suitable for screening protein-DNA interactions, including those that could be difficult with bulkier probes. This diazirine moiety can also be introduced directly on the surface of cells using sugar-derived probes, which are incorporated in glycosylated membrane proteins (Tanaka and Kohler, 2008). Recently, using Francis' allylpalladium chemistry on proteins, a fluorescent probe has been attached to a protein through a tyrosine residue. This molecule provided a probe for exploring the polarity of the environment around the labeled tyrosine of super oxide dismutase, as well as monitoring the change in polarity upon denaturation (Chen et al., 2009).

## Conclusion

Diversification of chemical transformations is still needed in order to apply a more versatile approach to proteome and interactome mapping. Selective modification of endogenous proteins remains a challenging task for the chemist. Two main facts can explain these difficulties. First, most chemistry developed during the last century was designed to obtain a specific product

and thus was not focused on the various reactivities potentially displayed by a reactant. As such, few chemical transformations are simultaneously compatible with all functionalities present on proteins. Therefore, the necessary equilibrium between reactivity and selectivity is difficult to maintain, particularly in the presence of water. The second main problem of protein modification is related to the potential polyfunctionalization of proteins. Reactions on C or N termini are by design selective. In contrast, for other reactions, many identical residues are present on the surface of a protein and obtaining a selective monofunctionalization of a protein is an arduous task. Additionally, once a protein has been modified, its properties can change drastically, and these changes in solubility or conformation can lead to cross-reactions, precipitation, or polyfunctionalization. These changes can also lead to the modulation of a protein's activity; and though this is a drawback for random screening in functional assays, it is an advantage for enzymatic activity modulation/stabilization. Overall, the desired application drives the selection of the method.

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