Cleavable Linkers

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Bisaryl Hydrazones as Exchangeable Biocompatible Linkers**

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The selective enrichment of tagged molecules from complex biological mixtures is of primary importance in chemical biology and proteomics. One of the most widely applied enrichment methods is affinity purification on (strept)avidin beads with biotin as an affinity tag. This strategy takes full advantage of the high affinity of biotin for (strept)avidin ($K_a \approx 1.7 \times 10^{15}\,\text{M}^{-1}$).[1] However, the method is limited by the harsh, denaturing conditions required for elution, such as boiling in a buffer containing sodium dodecyl sulfate (SDS) or treatment with an 8 M solution of guanidine (pH 1.5). Under these conditions, protein structure and function are lost, and target proteins may be contaminated with proteins bound nonspecifically to the beads. Two strategies have been explored for elution under mild conditions: 1) weakening of the biotin–(strept)avidin interaction by modulation of the K_a value[2] and 2) introduction of a proteolytically[3] or chemically[4] cleavable linker. Although the first strategy does improve the release of biotinylated proteins from (strept)avidin beads, it adversely affects the stringency of the immobilization. The second strategy enables site-specific cleavage; however, premature cleavage has been reported, and cleavage conditions have no demonstrated compatibility with active biomolecules. Furthermore, the general applicability of the cleavable linkers is often limited by the need for multistep organic synthesis before implementation.

Hydrazones have been explored for the reversible conjugation[5] and labeling[6–8] of biomolecules. However, their application has been limited, as most hydrazones are prone to hydrolysis and premature cleavage, whereas hydrazones (and oximes) that are fully stable under biological and mildly acidic conditions undergo slower hydrolysis and are difficult to exchange or cleave. In principle, a molecular catalyst could be used to accelerate hydrolysis and enable an effective transition between a stable state for workup and a dynamic state for cleavage and exchange.

Herein, we report that stable, yet exchangeable, bisaryl hydrazone linkers can be cleaved under mild conditions by catalytic transimination (Figure 1). The mechanism of transimination[9] enables the introduction of a new label to trace the originally biotinylated protein, or a new affinity tag for further purification (Figure 1 B). Alternatively, the biotin fragment bound to the beads can be capped with a benzaldehyde derivative to elute a protein with a synthetic handle that can be modified at a later time (Figure 1 C).

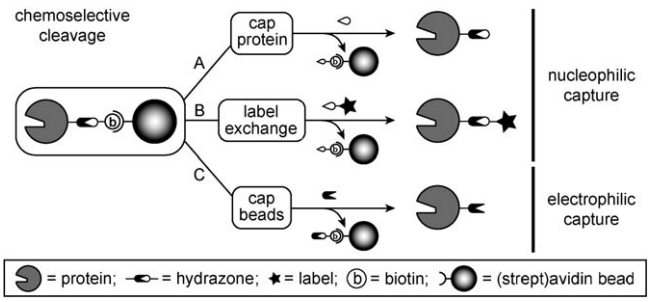

Figure 1. Three distinct strategies for the cleavage of a hydrazone linker: A) capping of the protein, B) label exchange for further analysis or purification, and C) capping of the beads to leave a synthetic handle on the protein for further modification.

The bisaryl hydrazone formed between a benzaldehyde and a hydrazinopyridine group has been successfully applied as a stable linker in bioconjugation and biomolecular labeling.[3a,10,11d] Notably, it has been included as a stable component of biotinylated protease-cleavable linker[3a] and as a stable chromogenic biotinylation agent.[10] The nucleophilic catalyst aniline accelerates effectively hydrazone formation and hydrolysis,[11] and an enhancement of the rate constants by two orders of magnitude is possible without a change in the pH value.[11d] As a result, the stable bisaryl hydrazone will become dynamic upon addition of the catalyst, which will make the bond prone to exchange and cleavage.

Indeed, a mixture of two bisaryl hydrazones (100 μM each) was stable at pH 7.0 (> 22 h), but rapidly reequilibrated in the presence of 100 mM aniline at pH 7.0 (< 8 h) to give four hydrazones (50 μM each; see Figure 1 in the Supporting Information). Reequilibration proceeded with the rate constant of catalyzed hydrolysis, which suggests that scrambling occurs through a direct reaction of trace free hydrazine and free aldehyde present in solution at equilibrium. Surprisingly, despite considerable analysis of the dynamic covalent chemistry of hydrazones,[12] hydrazone reequilibration under physiological conditions in the absence of excess monomers has not been reported previously.

The ability to convert the hydrazone bond effectively between stable and dynamic states is key for its use as an

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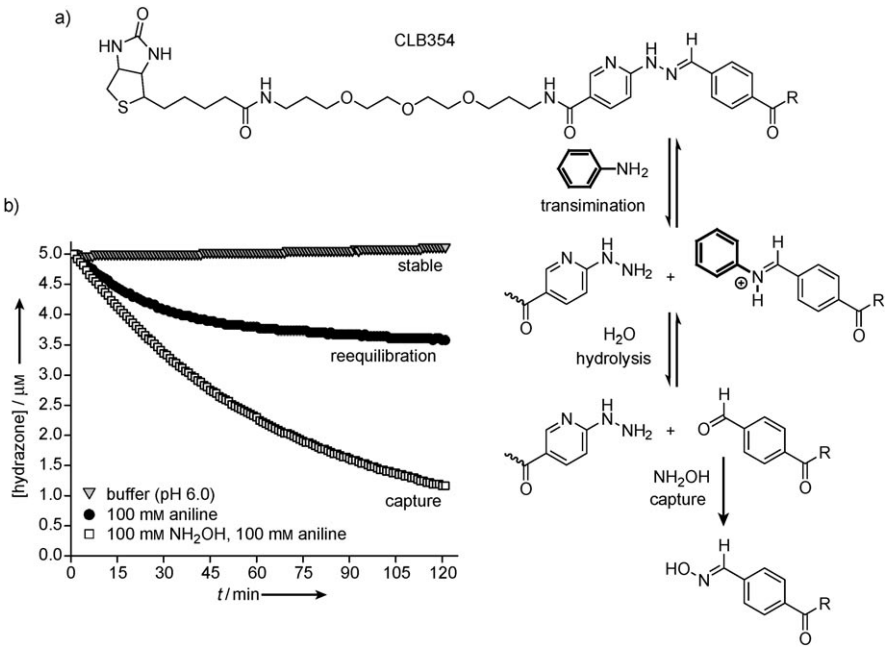

Figure 2. a) Transimination of biotinylation agent CLB354 with aniline in the presence of NH_2OH.[10] b) Graph showing the concentration of CLB354 (initial concentration: 5 μM) in sodium phosphate buffer (0.1 M, pH 6.0) with respect to time in the absence of amines (stable), in the presence of aniline (100 mM, reequilibration), and in the presence of NH_2OH (100 mM) and aniline (100 mM, capture).

exchangeable linker in pull-down assays for the enrichment and purification of proteins. The biotinylation agent CLB354 (Figure 2a) is commercially available, and its hydrazone linker has a distinct absorption in the visible region.[10] The reversibility and stability of CLB354 were first validated in solution at pH 6.0 to facilitate both reequilibration kinetics and protein stability. The CLB354 hydrazone (5 μM) was found to be fully stable overnight; however, upon treatment with 100 mM aniline, approximately 25% of the hydrazone was hydrolyzed rapidly, and a new equilibrium was attained (Figure 2b). As the hydrazone has a K_{eq} value of approximately $10^6\,M^{-1}$ in aqueous buffer,[11d] it would need to be diluted significantly to shift its equilibrium toward the starting materials and disrupt the hydrazone bond. However, the equilibrium can be pushed toward cleavage by trapping the free aldehyde with an aminooxy compound, such as hydroxylamine (NH_2OH), to give a more stable oxime (Figure 2b).[11c,13] The cleavage kinetics depend on the concentration of NH_2OH, and a significant, but limited enhancement was observed upon the addition of 100 mM aniline (see Figure 4 in the Supporting Information). These results suggest that at a high concentration of NH_2OH (> 10 mM), direct nucleophilic attack on the CLB354 hydrazone competes with aniline-catalyzed transimination. At 100 mM NH_2OH and 100 mM aniline, cleavage was quantitative within 8 hours (see Figure 5 in the Supporting Information).

The mechanism of cleavage is expected to be the same in solution as on (strept)avidin beads. However, the kinetics of the transimination reaction may be affected by the accumulation of free hydrazine groups on the beads as the reaction proceeds, and by the local environment created by the proteins. To gain insight into the cleavage efficiency on beads, we performed a model protein study in which biotinylated human serum albumin, HSA-CLB354$_x$ (8 μM; x is the average number of CLB354 molecules per HSA molecule), was retrieved from a well-defined protein mixture consisting of aprotinin, cytochrome *c*, myoglobin, and aldolase (10 μM each) in a solution at pH 7.4 containing sodium phosphate (50 mM) and NaCl (150 mM). HSA-CLB354$_x$ ($x = 1.3$) was pulled down from the protein mixture with avidin beads. The beads were washed and incubated for 2 h at pH 6.0 with varying amounts of NH_2OH and aniline (Figure 3) to elute HSA by capping the protein (Figure 1A). As reflected by SDS-PAGE analysis, the cleavage efficiency was improved, in accordance with the solution study, by increasing the concentration of NH_2OH as well as by the addition of aniline.

A hydrazone can be cleaved by transimination with any aminooxy compound. This approach offers the possibility to refunctionalize HSA by exchanging the biotin tag with a new label or affinity tag (Figure 1B). To demonstrate this principle, we cleaved the hydrazone of immobilized HSA-CLB354$_x$ ($x =$ 1.3) in a second experiment by using a cleavage buffer containing 10 mM aminooxyacetyl-Alexa Fluor 488 or aminooxyacetyl-modified FLAG peptide and 100 mM aniline (Figure 3). As reference experiments, the hydrazone was cleaved with 100 mM NH_2OH in the presence and absence of 100 mM aniline (see the Supporting Information), by incubation with just the cleavage buffer (0.1M sodium phosphate, pH 6.0), and by boiling in SDS-containing buffer (Figure 3). Equal amounts of beads were used in each experiment. The beads were incubated overnight, except in the case of heating in SDS-containing buffer, and the cleavage efficiencies were determined by densitometry (NIH Image J software). In comparison with the result of the use of SDS under harsh conditions, 74% of the immobilized HSA was recovered with 100 mM NH_2OH in the absence of aniline, and the recovery of immobilized HSA was increased to 88% in the presence of aniline. The biotin tag was exchanged effectively with the fluorescent Alexa dye, and 63% HSA–Alexa Fluor 488 was retrieved. Exchange with the FLAG tag appeared quantitative. Importantly, no detectable levels of HSA were observed when the beads were incubated with the buffer alone; thus, the hydrazone is completely stable at pH 6.0.

In all studies in which hydrazones have been used as cleavable functional groups, competing amines have been used for cleavage.[5–8] As the use of strongly nucleophilic amines may be incompatible with the function of certain proteins, we explored an alternative strategy involving the use

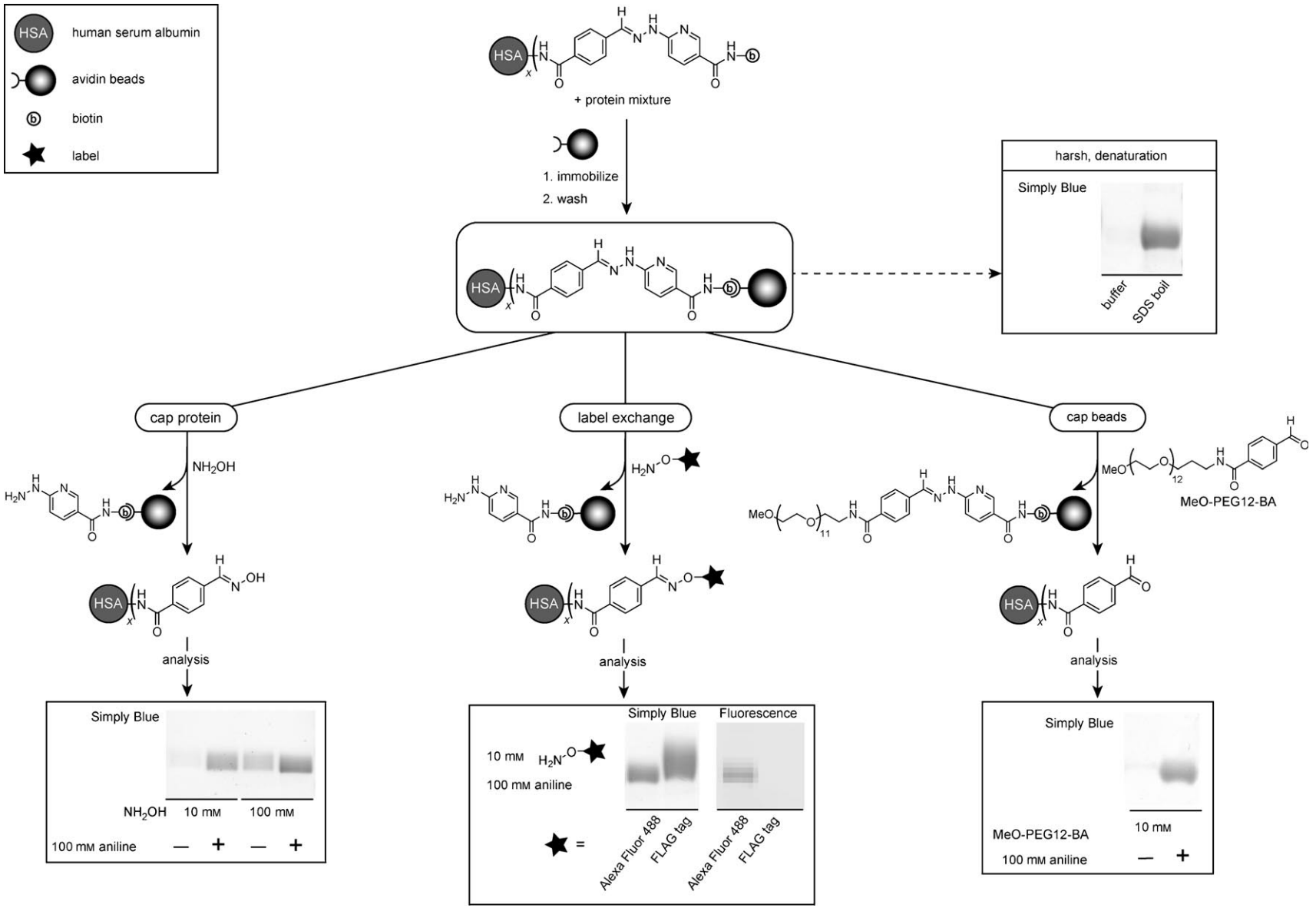

Figure 3. Retrieval of CLB354-labeled human serum albumin (HSA) from a well-defined protein mixture. The hydrazone was cleaved by capping the aldehyde on HSA with NH_2OH (bottom, left) or the hydrazinopyridine group on biotin with PEGylated benzaldehyde (bottom, right). Fluorescently labeled HSA was obtained by cleavage with aminooxyacetyl-Alexa Fluor 488, whereas a FLAG tag could be introduced by cleavage with aminooxyacetyl-FLAG (bottom, middle). Aniline catalyzes the transimination and improves cleavage efficiencies. SDS-PAGE analysis (4–12% Bis-Tris gel, Simply Blue stain; Bis-Tris = 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol) showed modified HSA. The hydrazone linker is stable in the buffer at pH 6.0 (top, right). CLB354-labeled HSA was treated with SDS in a boiling buffer for reference (top, right). No detectable levels of the other proteins in the protein mixture were observed after elution. See the Supporting Information for details.
PEG = poly(ethylene glycol).

of an aldehyde to capture the hydrazinopyridine group of the biotin fragment (Figure 1C). In contrast to cleavage with aminooxy compounds, this strategy requires aniline as a catalyst for transimination, as the aldehyde itself is electrophilic and unable to react directly with the hydrazone bond. For solubility reasons, a PEGylated benzaldehyde was used to cleave immobilized HSA–CLB354$_x$ ($x = 1.5$) instead of benzaldehyde. Indeed, in the absence of aniline, treatment with PEGylated benzaldehyde at a concentration of 10 mM overnight did not result in detectable cleavage of the hydrazone (Figure 3). However, upon the addition of aniline (100 mM), transimination occurred, and HSA–benzaldehyde was retrieved in about 60% yield (Figure 3), whereas PEGylated biotin remained on the beads. Importantly, in the released benzaldehyde-functionalized protein, a new synthetic handle is available for further modification.

Having fully demonstrated the scope of the method in model systems, we implemented CLB354 in our ongoing research program aimed at the discovery of new binding partners of anticoagulant protein S, the known binding partner of which is complement factor protein C4bP. Recent studies have suggested that protein S might have several other functions besides its well-known activated protein C (APC) cofactor activity.[14–16] Owing to the newly discovered functions of protein S, there has been renewed interest in the discovery of new binding partners in plasma and on cells.[17] We synthesized protein S–CLB354$_{3.4}$ and used it in a pull-down assay to retrieve its binding partners from human plasma. Protein S–CLB354$_{3.4}$ and its binding partners were pulled down with avidin beads. The beads were washed, and the remaining proteins were eluted overnight with a solution at pH 6.0 containing NH_2OH (100 mM) and aniline (100 mM). Approximately 80% of the proteins immobilized on the beads were eluted by this procedure (see the Supporting Information). The proteins were analyzed by SDS-PAGE and western blotting. As anticipated, C4bP was enriched, and a potential new binding partner of protein S was discovered (see the Supporting Information).

It is anticipated that the method presented herein is compatible with a wide range of biomolecules, and the mild cleavage conditions should enable the retrieval of active, structurally intact proteins. Indeed, protein S was found to maintain full APC cofactor activity after incubation overnight under our harshest cleavage conditions: treatment with NH_2OH (100 mM) and aniline (100 mM) at pH 6.0 (see Figure 15 in the Supporting Information). Alternatively, proteins that are incompatible with strong nucleophiles can be cleaved with an aldehyde in the presence of aniline. The method is highly flexible and can be optimized to meet the conditions and requirements of individual systems. Cleavage times can be reduced significantly by lowering the pH value, by increasing the temperature, or by increasing the concentration of the nucleophile (aniline, aminooxy groups) in the cleavage cocktail. For example, the bisaryl hydrazone can be cleaved quantitatively within 1 h with NH_2OH (100 mM) in 0.1M anilinium acetate (pH 4.6; see Figure 8 in the Supporting Information). The method further distinguishes itself from existing methods by offering the opportunity to introduce a new label or affinity tag, or to preserve a synthetic handle for further modification. These results suggest that detailed analysis of the kinetics and thermodynamics of hydrazones and oximes could expand the utility of these groups in chemical bioapplications.

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