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Rapid Oxime and Hydrazone Ligations with Aromatic Aldehydes for Biomolecular Labeling

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Abstract

A high yielding and rapid chemoselective ligation approach is presented that uses aniline catalysis to activate aromatic aldehydes towards two amine nucleophiles, namely 6-hydrazinopyridyl and aminooxyacetyl groups. The rates of these ligations are resolved for model reactions with unprotected peptides. The resulting hydrazone and oxime conjugates are attained under ambient conditions with rate constants of 10^1 – 10^3 M⁻¹s⁻¹. These rate constants exceed those of current chemoselective ligation chemistries and enable efficient labeling of peptides and proteins at low μ M concentrations, at neutral pH, without using a large excess of one of the components. The utility of the approach is demonstrated by the *p*-fluorobenzoylation of Human Serum Albumin and by the fluorescent labeling of an unprotected peptide with Alexa Fluor 488.

Keywords

bioconjugation; labeling; hydrazone; oxime ligation; nucleophilic catalysis

Introduction

Chemoselective ligation chemistries facilitate the site-specific modification and assembly of biomolecules.(1-3) However, the growing demands of research in diverse areas of chemical biology have pushed the limits of current ligation methods, especially in terms of ligation kinetics.(3) Since biomolecules are often present at low concentrations, their efficient labeling depends on the reaction rate of the ligation chemistry used. The reaction rate constants of current chemoselective ligation chemistries typically range from 10^{-3} – 10^0 M⁻¹s⁻¹ under ambient conditions.(3-12) As a result, high concentrations, a large excess of one of the reactants or elevated temperatures are typically used to compensate for the slow reaction kinetics.

Imine ligations have found significant utility in biomolecular labeling.(13-25) The reaction between an amine and a carbonyl is highly chemoselective and is compatible with other functional groups present in biomolecules. The resulting imine bond is covalent but reversible, and both its stability and dynamics strongly depend on the nature of the amine and the carbonyl. In general, aldehydes are substantially more reactive than ketones, mainly due to steric effects.(26) Under aqueous conditions, α -effect amines (hydrazines and aminooxy groups) react to form hydrazones and oximes.(11-25) The equilibrium constant (K_{eq}), the ratio between the rate constant of formation k_1 and the rate constant of hydrolysis k_{-1} , is typically in the range of 10^4 – 10^6 M⁻¹ for hydrazone linkages and $>10^8$ M⁻¹ for oxime bonds. The importance of K_{eq} comes to light when imine ligations are performed at low concentration (low μ M range)

or when a bioconjugation product is stored at sub- μM concentration. When the K_{eq} is relatively small, as is the case for many hydrazone linkages, the ligation reaction will not go to completion (100% product). Similarly, if a ligation product is stored under too dilute conditions, the system will eventually reequilibrate and the conjugate will partially dissociate again.

Inspired by the early work of Cordes and Jencks,(27,28) we recently reported aniline as a potent nucleophilic catalyst for imine ligation and transimination.(11,12) These studies focused on the commonly used glyoxylyl group as the electrophile, and aniline catalysis enhanced the rates of these slow reactions to rates comparable to those of other widely used chemoselective ligation chemistries, including the Staudinger ligation,(4-6) the Cu(I)-catalyzed [3+2] azide-alkyne cycloaddition,(7,8,29) the Cu(I)-free click chemistry,(9) and native chemical ligation.(2,10,30) However, these reactions (10^{-3} - $10^0 \text{ M}^{-1}\text{s}^{-1}$) are many orders of magnitude slower than enzymatic labeling methods,(31) such as the HaloTagTM technology ($\sim 10^6 \text{ M}^{-1}\text{s}^{-1}$),(32) noncovalent binding events such as protein-protein interactions ($\sim 10^5 \text{ M}^{-1}\text{s}^{-1}$)(33) or the biotin-streptavidin interaction ($\sim 10^6 \text{ M}^{-1}\text{s}^{-1}$)(34). The current limitations are illustrated in Figure 1, which shows simulated 2nd order reactions at 100 μM reactants. Even with a rate constant of $1 \text{ M}^{-1}\text{s}^{-1}$, one needs over 1 day to achieve > 90% conversion. As a result, efficient ligation requires the use of higher concentrations, a large excess of one of the reactants, or elevated temperatures. This analysis suggests that for rapid ligation at 100 μM of each reactant, reaction rate constants of greater than $10 \text{ M}^{-1}\text{s}^{-1}$ need to be achieved.

In this light, we further explored the scope of aniline-catalyzed imine chemistry by investigating the reaction between aromatic aldehydes and either 6-hydrazinopyridine or aminooxyacetyl groups. Aromatic aldehydes are potent electrophiles and are more reactive than the ketone and glyoxylyl groups typically used in imine bioconjugation reactions. Aminooxyacetyl groups are readily incorporated into biological macromolecules and chemoselectively form a hydrolytically stable oxime bond, yet the kinetics are slow at neutral pH. An alternative group, 6-Hydrazinopyridine readily forms a hydrazone linkage with aromatic aldehydes to give a chromophore ($\lambda_{\text{max}} \sim 350 \text{ nm}$) that enables direct spectroscopic monitoring of the reaction by UV-Vis.(35) 6-Hydrazinopyridine has similar reactivity to phenylhydrazine, but is less prone to oxidation side reactions.(36) The 6-hydrazinopyridyl group can be readily incorporated into biomolecules using 6-hydrazinonicotinic acid, also known as HYNIC, a widely used ligand for ^{99m}Tc in radiolabeling.(36) The ligation between aromatic aldehydes and 6-hydrazinopyridine has been applied to biomolecular labeling,(37-40) but the kinetics of the reaction have not been reported.

Experimental Procedures

Solvents and starting materials

Unless stated otherwise, all reagents and solvents were purchased from commercial sources and used without purification.

Note on the stability of 6-hydrazinopyridine and its hydrazone with benzaldehyde

In contrast to the hydrazone, the stability of the free hydrazinopyridine is limited. As a solid, the compound is best stored in the freezer protected from light as a salt (HCl / TFA). In solution, the hydrazinopyridine moiety is prone to decomposition due to oxidation in a similar way, but to a lesser extent than phenylhydrazine.(36) The fast ligation rates observed in the presence of aniline minimize these side reactions. HYNIC labeled molecules are stored at + 4 °C or -20 °C, under nitrogen and protected from light. Hydrazone products based on HYNIC and benzaldehyde are stored at a concentration of > 50 μM .

Instrumentation

Reversed phase high pressure liquid chromatography (RP HPLC) was performed on a HP1050 HPLC System (analytical HPLC) and on a Waters Delta Prep 4000 preparative chromatography system (preparative HPLC), using a Phenomenex Prodigy 5 μ 100Å (50 $\times$ 4.60 mm) and a Phenomenex Jupiter 10 μ Proteo 90Å (250 $\times$ 21.2 mm), respectively, for separation. Electrospray ionization mass spectrometry (ESI-MS) was performed on a SCIEX API-I single quadrupole mass spectrometer. UV-Vis was measured on a GenesysTM 6 spectrophotometer (Thermo Electron Corporation).

Peptide synthesis

The peptides were obtained *via* manual solid phase peptide synthesis (SPPS) using the *in situ* neutralization/1*H*-benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate-(1-),3-oxide (HCTU) activation procedure for *t*Boc chemistry on a *p*-methylbenzhydrylamine (MBHA) resin.(41) Detailed procedures are given in the Supporting Information.

Hydrazone ligations between 6-hydrazinopyridyl-peptide 1 and benzaldehyde

A 2 mM stock solution of HYNIC-peptide **1** (A), a 2 mM stock solution of benzaldehyde (B), and a 200 mM stock solution of aniline (C) were freshly prepared in either 0.1 M NH₄OAc (pH 4.5) or 0.3 M Na phosphate (pH 7). The stock solutions were mixed to give the desired concentration of **1**, benzaldehyde, and aniline in the final reaction mixture. The reactions at 100 μ M were followed by RP HPLC (220 nm, 350 nm) and the reactions at 10 μ M were followed by UV-Vis (350 nm). Hydrazone product **2** was analyzed by ESI-MS. **2**: ESI-MS calcd. for C₆₈H₉₃N₂₀O₂₅ ([M+H]⁺): 1591.6, found 1591.5 $\pm$ 0.7. The data were fitted to the solution of the rate equation of a 2nd order (reversible) reaction. Details on the kinetics experiments are given in the Supporting Information.

Labeling of HYNIC-HSA with *p*-¹⁹F-benzaldehyde

For the labeling of HYNIC-HSA with *p*-¹⁹F-benzaldehyde in the absence of aniline, 350 μ L of 0.1 M Na phosphate (pH 7) and 50 μ L of a 2.16 mM solution of *p*-¹⁹F-benzaldehyde in 0.1 M Na phosphate (pH 7) were subsequently added to 100 μ L of a HYNIC-HSA stock solution ([HSA] = 150 μ M, on average ~6 HYNIC/HSA, see Supporting Information). The reaction mixture was vortexed and added into a quartz cuvette. The reaction was followed by UV-Vis (370 nm). The final concentration of HYNIC-HSA was 30 μ M (2 mg/mL). This concentration of HYNIC-HSA was also used by Chang *et al.* in a typical procedure for ¹⁸F labeling with *p*-¹⁸F-benzaldehyde.(40)

For the labeling of HYNIC-HSA with *p*-¹⁹F-benzaldehyde in the presence of 100 mM aniline, 200 μ L of 0.1 M Na phosphate (pH 7), 100 μ L of a 2.16 mM solution of *p*-¹⁹F-benzaldehyde in 0.1 M Na phosphate (pH 7), and 500 μ L of a 200 mM stock solution of aniline in 0.1 M Na phosphate (pH 7) were subsequently added to 200 μ L of a HYNIC-HSA stock solution ([HSA] = 150 μ M, on average ~6 HYNIC/HSA). The reaction mixture was vortexed and added into a quartz cuvette. The reaction was followed by UV-Vis (370 nm). After 1 hour, the excess of *p*-¹⁹F-benzaldehyde and the aniline catalyst were removed by size-exclusion chromatography over a NAPTM 5 column. The stability of the hydrazone ligation product was verified by measuring the absorption at 370 nm. No decrease in the absorption of the hydrazone was observed over a period of 20 hours.

Labeling of benzaldehyde-peptide 4 with aminooxyacetyl-Alexa Fluor® 488 (10 μ M reactants)

Aminooxyacetyl-Alexa Fluor® 488 is commercially available and is delivered in a vial containing 1 mg of the dye. A stock solution of approx. 1 mM in water was made by adding

1.117 mL of water (A). In addition, a 100 μ M stock solution of the **4** (B) and a 200 mM stock solution of aniline (C) in 0.1 M Na phosphate (pH 7) were prepared. For the reaction in the absence of aniline, 200 μ L of B and 20 μ L of A were subsequently added to 1.780 mL of 0.1 M Na phosphate (pH 7). For the reaction in the presence of 100 mM aniline, 1.000 mL of C, 200 μ L of B, and 20 μ L of A were subsequently added to 780 μ L of 0.1 M Na phosphate (pH 7). The reactions were followed by RP HPLC (220 nm, 495 nm; gradient: 5 - 25% 9:1 v/v MeCN/H₂O in H₂O, 0.1 v-% TFA in 20 min; flow: 3 mL/min). The exact concentration of dye in the ligation reactions was determined by measuring the absorption at 495 nm ($\epsilon = 7.1 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) and was calculated to be 13 μ M. Oxime product **5** was quantitated by integration (495 nm) and analyzed by ESI-MS. **5**: ESI-MS calcd. for C₆₄H₇₇N₁₅O₂₀S₂ ([M+H]⁺): 1441.5, found 1442.0.

Results and discussion

To evaluate the utility of combining aniline catalysis with arylaldehyde electrophiles, we first reacted 6-hydrazinopyridyl peptide **1** and benzaldehyde (Figure 2a) (100 μ M each) at pH 4.5, near the pH optimum of imine reactions. The reaction was followed by HPLC (220 nm, 350 nm) and the data were fitted to the rate equation of a 2nd order reaction. Full conversion to hydrazone product **2** was reached within a day with $k_1 = 3.0 \pm 0.3 \text{ M}^{-1}\text{s}^{-1}$ (see Supporting Information), much faster than typical imine ligations used in bioconjugation and matching the fastest chemoselective ligation reactions in the literature. Furthermore, in the presence of 10 mM aniline the reaction reached full conversion within minutes under the same conditions. To fully characterize the k_1 , k_{-1} , and K_{eq} of this reaction in the absence and presence of aniline, 6-hydrazinopyridyl-peptide **1** and benzaldehyde were reacted under more dilute conditions. The reactions were followed by absorbance at 350 nm. At 10 μ M reactants, the reaction reached an equilibrium with ~60% product present (Figure 2b). In accordance with the experiment at 100 μ M reactants, the k_1 of the uncatalyzed reaction was $2.6 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$. The k_{-1} was found to be $(0.03 \pm 0.001) \times 10^{-3} \text{ s}^{-1}$, giving a K_{eq} of $(0.88 \pm 0.04) \times 10^6 \text{ M}^{-1}$. In the presence of 10 mM aniline, k was enhanced over 70-fold to $190 \pm 10 \text{ M}^{-1}\text{s}^{-1}$ and k_{-1} increased accordingly to $(0.81 \pm 0.03) \times 10^{-3} \text{ s}^{-1}$, giving a $K_{eq} = (0.23 \pm 0.02) \times 10^6 \text{ M}^{-1}$. An additional 10-fold rate enhancement was achieved by using 100 mM aniline: $k_1 = 2,000 \pm 100 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = (5.1 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$, and $K_{eq} = (0.40 \pm 0.02) \times 10^6 \text{ M}^{-1}$. Despite the very dilute conditions, the reaction reached equilibrium within minutes (Figure 2b).

Many biomolecules are conformationally unstable under acidic conditions and at the same time limited to low concentrations (μ M range) owing to their high molecular weight, low availability, and tendency to aggregate. Biomolecular labeling at pH 7 at low concentrations is therefore often desired. At pH 7 the reaction between 6-hydrazinopyridyl-peptide **1** and benzaldehyde (10 μ M each) rapidly reaches equilibrium in the presence of 100 mM aniline; $k_1 = 170 \pm 10 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = (0.08 \pm 0.004) \times 10^{-3} \text{ s}^{-1}$ and the equilibrium shifts slightly more towards the product (~80% product; $K_{eq} = (2.3 \pm 0.1) \times 10^6 \text{ M}^{-1}$) (see Supporting Information). To the best of our knowledge, the aniline-catalyzed reaction between the 6-hydrazinopyridyl group and benzaldehyde is the fastest chemoselective ligation chemistry that has been described.

The K_{eq} of this reaction implies that at least one of the components should be present at $> 50 \mu$ M in order to shift the equilibrium completely ($> 99\%$) towards the product and at $> 13 \mu$ M to reach $> 90\%$ conversion. When labeling is complete and the excess of one of the components is removed, a new equilibrium will be reached over time, determined by the k_{-1} of the reaction. Consistent with its role as a nucleophilic catalyst, aniline does not affect K_{eq} , but does catalyze reequilibration. However, when the catalyst is removed during work-up, reequilibration would take weeks ($k_{-1} \sim 10^{-6} \text{ s}^{-1}$ at pH 7).

The rapid ligation between the 6-hydrazinopyridyl group and benzaldehyde in the presence of aniline may be of particular use for radiolabeling, since the time of labeling is strongly limited by the half-life of the radiolabel. Recently, a p - ^{18}F -benzaldehyde label was developed for positron emission tomography (PET) imaging.(42) However, high temperatures (50–60 °C) (40,42) and low pH(42) were required to compensate for the slow ligation rates and to achieve optimal labeling within typical reaction times of 10–30 minutes. To demonstrate the impact of aniline catalysis, the labeling of 6-hydrazinopyridyl-functionalized Human Serum Albumin (HYNIC-HSA) with p - ^{19}F -benzaldehyde was performed in the absence and presence of 100 mM aniline, using only a slight excess (1.2 equiv per HYNIC) of the label at room temperature (Figure 3a). For this, HSA was functionalized with on average 6 HYNIC moieties (see Supporting Information). Labeling was performed at 30 μM HYNIC-HSA, a concentration used in a recently reported optimized protocol for p - ^{18}F -benzaldehyde labeling.(40)

As shown in Figure 3b, the labeling of HYNIC-HSA with p - ^{19}F -benzaldehyde is relatively slow. Less than 10% labeling is achieved within 30 minutes, consistent with the use of elevated temperatures and acidic conditions in previous labeling protocols for p - ^{18}F -benzaldehyde. In the presence of aniline, the reaction kinetics are strongly enhanced and > 95% labeling is established within the same amount of time at neutral pH, at room temperature. After removal of the aniline catalyst and the slight excess of label by size exclusion (NAPTM 5 column), the hydrazone linkage was found to be stable for at least 20 hours at room temperature.

In typical radiolabeling experiments with p - ^{18}F -benzaldehyde, the label is only present in high nM to low μM concentrations, while the biomolecule is in great excess (high μM to low mM). Under these conditions, the labeling reaction will be pseudo-first order in the biomolecule. If the reaction described above would be performed with the same initial concentration of HYNIC-HSA (6 HYNIC per HSA on average), but with a much lower concentration of p - ^{19}F -benzaldehyde, this pseudo-first order reaction would actually reach completion more rapidly than the second order reaction where an equimolar amount of p - ^{19}F -benzaldehyde is applied.

Since oximes are more hydrolytically stable than hydrazones, we investigated the combination of the aminooxy nucleophile with benzaldehyde electrophiles. To gain insight into the kinetics of the aniline-catalyzed oxime ligation, aminooxyacetyl-peptide **3** and benzaldehyde (100 μM each) were reacted under ambient conditions at pH 7 in the presence of 100 mM aniline (Figure 4). Although the k_1 of $8.2 \pm 1.0 \text{ M}^{-1}\text{s}^{-1}$ is 20-fold smaller than the k_1 of the reaction between the 6-hydrazinopyridyl group and benzaldehyde, the K_{eq} of the oxime is at least 2 orders of magnitude larger than the K_{eq} of the hydrazone. This will enable quantitative conjugation at 10 μM within 1 day using only a slight excess of label.

To demonstrate the utility of oxime ligation for labeling, benzaldehyde-functionalized peptide **4** (10 μM) was tagged with the fluorescent label aminooxyacetyl-Alexa Fluor® 488 at pH 7 (Figure 5a). As anticipated, labeling was established within 1 day, whereas in the absence of aniline < 5% conversion was reached within the same period of time (Figure 5b).

Conclusions

Many methods are available to introduce benzaldehyde, 6-hydrazinopyridyl (HYNIC), or aminooxyacetyl groups in a random or site-specific manner into biomolecules.(1–3,35,36) Aniline catalysis has given the reaction between aromatic aldehydes and α -effect amines a new dimension by exceeding the reaction rates of current chemoselective ligation chemistries and by bringing covalent chemistry one step closer to the rates observed in non-covalent interactions.

Aniline is a weak base that is moderately nucleophilic and does not react with the amino acid side chains of unmodified peptides. Aniline can be readily removed from the conjugation mixture by HPLC or size exclusion chromatography. Aniline may act as a competitive ligand for some metalloproteins as does imidazole,(43) a widely used acid-base and nucleophilic catalyst. In addition, aniline will form a reversible Schiff base with aldehyde-containing proteins, which may affect their function. However, for most proteins aniline acts as an organic buffer and is not expected to affect binding or catalysis.(44) Therefore, we anticipate that the aniline-catalyzed imine chemistry presented in this paper will find wide utility in biomolecular labeling owing to its fast kinetics under mild conditions at neutral pH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Simulation of 2nd order reactions between 100 μ M reactants showing the formation of product in time as a function of the reaction rate constant. Rate constants of current chemoselective ligation chemistries typically range from 10^{-3} - $10^0 \text{ M}^{-1}\text{s}^{-1}$.

Figure 2.

(a) Hydrazone reaction of 6-hydrazinopyridyl-peptide **1** and benzaldehyde in the absence and presence of aniline catalyst; (b) Formation of hydrazone **2** over time at $10 \mu\text{M}$ reactants in $0.1 \text{ M NH}_4\text{OAc}$ (pH 4.5) at room temperature in the absence ($\blacktriangledown$) and in the presence of 10 mM ($\bullet$) and 100 mM ($\blacksquare$) aniline. The dotted lines represent the fit of the data to the rate equation (full data for $\blacktriangledown$ is included in Supporting Information Figure 2).

Figure 3.

(a) Hydrazone reaction of 6-hydrazinopyridyl-functionalized Human Serum Albumin (HYNIC-HSA; 6 HYNIC per HSA on average) and p - ^{19}F -benzaldehyde in the absence and presence of aniline catalyst; (b) Labeling of HYNIC-HSA followed in time by UV-Vis (370 nm) in the absence (○) and in the presence (●) of 100 mM aniline; [HYNIC-HSA] = 30 μM , [p - ^{19}F -benzaldehyde] = 216 μM , 0.1 M Na phosphate (pH 7), room temperature.

Figure 4.

(a) Oxime ligation of aminooxyacetyl-functionalized peptide **3** and benzaldehyde in the presence of aniline catalyst; (b) Formation of oxime **3a** over time at 100 μM reactants in 0.3 M Na phosphate (pH 7) at room temperature in the presence of 100 mM aniline. The dotted line represents the fit of the data to the rate equation.

Figure 5.

(a) Oxime ligation of aminooxyacetyl-Alexa Fluor® 488 and benzaldehyde-functionalized peptide **4** in the absence and presence of aniline catalyst; (b) Formation of oxime **5** over time reacting 10 μM of **4** with 13 μM (1.3 equiv) of aminooxyacetyl-functionalized Alexa Fluor® 488 in 0.1 M Na phosphate (pH 7) at room temperature in the absence ($\circ$) and in the presence ($\bullet$) of 100 mM aniline.