Oxime Ligation

DOI: 10.1002/ange.200602877

Nucleophilic Catalysis of Oxime Ligation**

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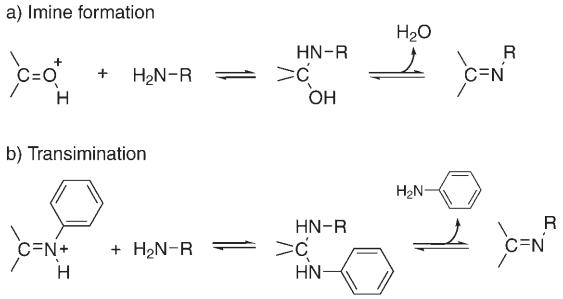
Imine-based reactions have found wide application in the conjugation of biomolecules as a result of their high chemoselectivity.[1–8] Aldehydes and ketones can be readily introduced into biomolecules and are virtually inert towards reaction with other functional groups in these molecules. Under acidic conditions the carbonyl group reacts with primary amines to form a reversible imine (Scheme 1a), and the equilibrium favors the free carbonyl. However, when α-effect nitrogens[9] such as aminooxy groups and hydrazides are used, the equilibrium favors the imine.[1–8]



Scheme 1. a) Imine formation and b) transimination under acidic conditions.

Oxime ligations are often called upon to link complex and precious macromolecules.[7,8] The oxime bond is stable under physiological conditions, whereas more dynamic imines, such as hydrazones, are often reduced to obtain a stable linkage. Oxime ligations proceed with modest reaction rates in acidic solution but are poorly reactive at pH 7. which limits their use in many biological applications. To improve the reaction rate, oxime ligations typically require millimolar concentrations of

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[**] This work was supported by the Netherlands Organization for Scientific Research (NWO; to A.D.) and by the National Institutes of Health (GM059380 to P.E.D.).

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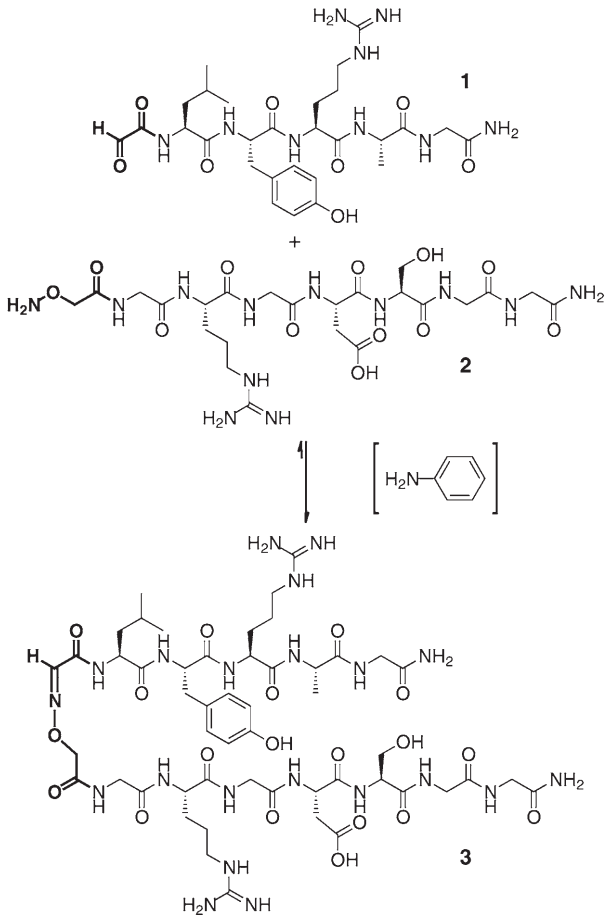
each reactant or a large excess of one component to establish pseudo-first-order kinetics.

Here we show that oxime ligations can be significantly accelerated by using aniline as a nucleophilic catalyst. Rate enhancements are achieved by changing the electrophile from a weakly populated protonated carbonyl (Scheme 1a) to a more highly populated protonated aniline Schiff base[10] (Scheme 1b).[11] The transimination ("trans-Schiffization")[11] of the protonated aniline Schiff base to the oxime proceeds rapidly under acidic aqueous conditions. The resulting ligation rates (k_{obs}) are increased up to 400-fold in aqueous solution at pH 4.5 and up to 40-fold at pH 7. Such rate enhancements enable the use of equimolar reactants at submillimolar concentrations, meeting the stringent requirements of challenging macromolecular ligation.

To illustrate the method, two unprotected peptides, glyoxylyl-LYRAG **1** and aminooxyacetyl-GRGDSGG **2** (Scheme 2) were ligated in the presence and absence of aniline at pH 4.5. The ligation reactions were performed at ambient temperature with each peptide at a concentration of 1 mM. The reactions were followed by HPLC, the ligation product **3** was quantitated by integration (214 nm), and the resulting data were fit to the second-order rate equation. In the absence of a catalyst, the half-time ($t_{1/2}$) of the reaction, that is, the time required to reach 50% conversion, is 310 min ($k_{obs} = 0.057 \pm 0.010\,M^{-1}s^{-1}$; Figure 1). However, in the presence of 10 mM aniline $t_{1/2}$ is reduced to 25 min ($k_{obs} = 0.68 \pm 0.05\,M^{-1}s^{-1}$). A further increase in the reaction rate is established by using a 100 mM anilinium acetate buffer (pH 4.6; Figure 1). Consistent with the proposed mechanism of nucleophilic catalysis, aniline does not change the equilibrium for the oxime ligation, and all reactions eventually reach over 99% conversion to **3**.



Scheme 2. Oxime ligation between glyoxylyl-LYRAG **1** and aminooxyacetyl-GRGDSGG **2** performed in either the absence or the presence of aniline.

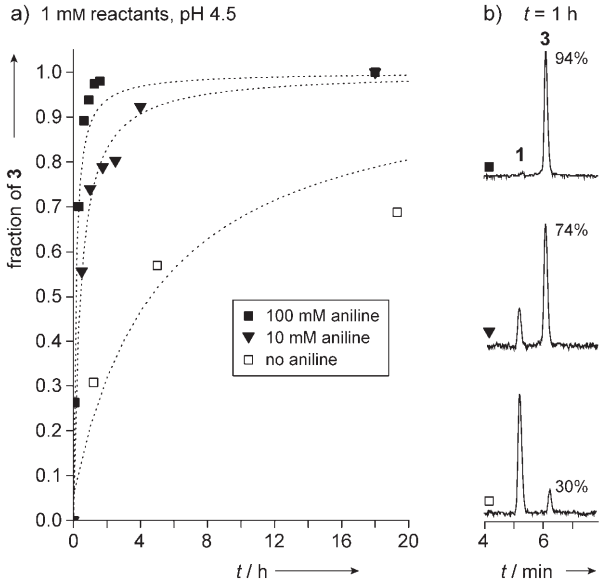


Figure 1. a) Formation of ligation product **3** over time at a reactant concentration of 1 mM in 0.1 M ammonium acetate buffer (pH 4.5) in the absence (□) and in the presence of 10 mM aniline (▼), and in 100 mM anilinium acetate buffer (pH 4.6; ■) ([**1**] = [**2**] = 1 mM; RT). b) RP HPLC traces recorded after 1 h for the three ligation reactions (gradient: 5–15% 9:1 v/v MeCN/H_2O in H_2O, 0.1% TFA in 10 min).

The solubility of large macromolecules is frequently limited to 0.1 mM concentrations owing to their high molecular weight, low availability, and tendency to aggregate. The enhanced ligation rates achieved through catalytic transimination suggested that oxime bonds could be formed efficiently at these low concentrations. Indeed, when the ligation is performed at pH 4.5 and at 0.1 mM of each peptide, the $t_{1/2}$ for oxime formation is reduced 30-fold from 8200 minutes (5.7 d; $k_{obs} = 0.020 \pm 0.001\,M^{-1}s^{-1}$) in the absence of aniline to 270 min ($k_{obs} = 0.62 \pm 0.03\,M^{-1}s^{-1}$) in the presence of 10 mM aniline (Figure 2). The ligation rate was further increased to a total of more than 400-fold using a 100 mM anilinium acetate buffer (pH 4.6); thus the peptides could be ligated with a $t_{1/2}$ of just 19 min ($k_{obs} = 8.6 \pm 2.0\,M^{-1}s^{-1}$) (Figure 2).

Since many biological macromolecules are conformationally unstable at low pH, it is often desirable to perform ligation reactions at pH 7. As shown in Figure 3, the uncatalyzed ligation is very slow under these conditions; $t_{1/2}$ is 11000 min (7.6 days; $k_{obs} = 0.0015 \pm 0.0001\,M^{-1}s^{-1}$), and competition is observed with the slow intramolecular ring closure of the glyoxylyl-functionalized peptide **1** to its corresponding cyclic isomers.[12] In contrast, oxime ligation

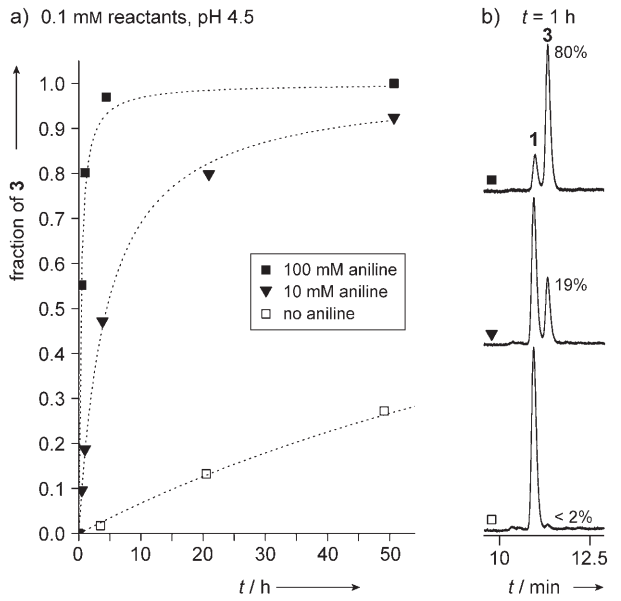


Figure 2. a) Formation of ligation product **3** over time at a reactant concentration of 0.1 mM in a 0.1 M ammonium acetate buffer (pH 4.5) in the absence (□) and in the presence of 10 mM aniline (▼), and in a 100 mM anilinium acetate buffer (pH 4.6; ■) ([**1**] = [**2**] = 0.1 mM; RT). b) RP HPLC traces recorded after 1 h for the three ligation reactions (gradient: 0–33.5 % 9:1 v/v MeCN/H_2O in H_2O, 0.1 % TFA in 15 min).

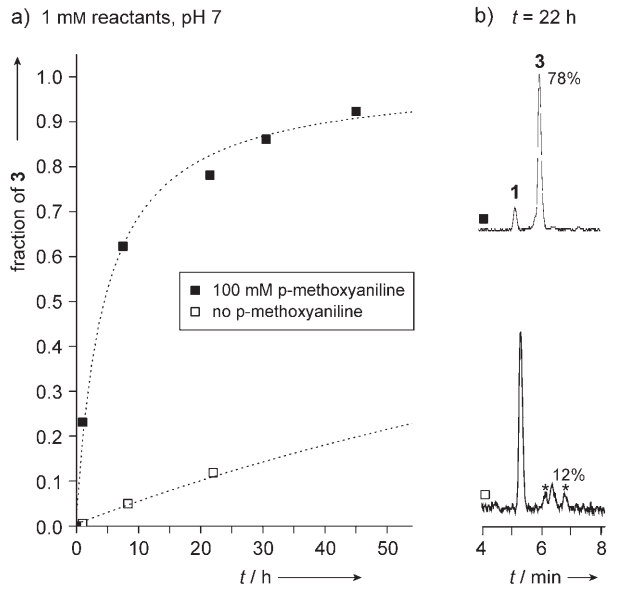


Figure 3. a) Formation of ligation product **3** over time at a reactant concentration of 1 mM in a 0.1 M sodium phosphate buffer (pH 7) in the absence (□) and in the presence of 100 mM *p*-methoxyaniline (▼) ([**1**] = [**2**] = 1 mM; RT). b) RP HPLC traces recorded after 22 h for the two ligation reactions (gradient: 5–15 % 9:1 v/v MeCN/H_2O in H_2O, 0.1 % TFA in 10 min); the peaks marked with an asterisk correspond to two isomers of the cyclization product of **1** (confirmed by ESI-MS).

at neutral pH can be achieved by using 100 mM *p*-methoxyaniline as a catalyst (selected because of its elevated pK_a of 5.3). In the presence of the *p*-methoxyaniline catalyst, the $t_{1/2}$ is 280 min ($k_{obs} = 0.061 \pm 0.006\ M^{-1}s^{-1}$; Figure 3), similar to the rate observed for the uncatalyzed oxime ligation at pH 4.5. These reaction conditions could find utility in the ligation or labeling of folded protein domains.

The application of aniline as a nucleophilic catalyst extends the scope of oxime ligations to new conditions highly relevant for bioconjugation. The rate enhancement can be attributed to the in situ generation of a comparatively high concentration of a reactive electrophile, the protonated aniline Schiff base, without affecting the equilibrium for oxime formation. It has been established that imine formation involves the nucleophilic attack of a free amine on the protonated carbonyl followed by dehydration (Scheme 1 a).[13,14] Since the pK_a of a protonated carbonyl is in the range of −4 to −10,[15] the concentration of this highly reactive electrophile is extremely low at pH 4.5. Although lowering the pH would lead to an increase in protonated carbonyl, the concomitant protonation of the reacting amine counteracts this effect.

Aniline and its derivatives meet the unique requirements for nucleophilic catalysis of oxime ligations.[11] Aniline (pK_a = 4.6; *p*-methoxyaniline: pK_a = 5.3)[11a] has a pK_a similar to that of α-effect nitrogens, such as the amine of the aminooxy group (p$K_a \approx 4.6$).[16] However, the pK_a of an imine formed with an α-effect nitrogen is typically 5–6 units below the pK_a of the parent amine, whereas the pK_a of the aniline Schiff base is only 2 units below that of aniline.[17] Consequently, the aniline Schiff base is significantly protonated under ligation conditions, whereas the imine formed with the α-effect nitrogen is almost completely deprotonated. In addition, the small equilibrium constant (K_{eq}) for the formation of the aniline Schiff base[11] allows the use of an excess of aniline without having it compete with the formation of the oxime. At neutral pH, aniline derivatives with a high pK_a should be more effective catalysts, establishing higher concentrations of the protonated aniline Schiff base. The ability to generate a reactive species without competing with the desired product is a key property of any nucleophilic catalyst.

Nucleophilic catalysis using aniline or other appropriate amine derivatives should be applicable to any reaction for which imine formation or transimination is the rate-determining step. Aniline is a mild, fairly unreactive nucleophile and is therefore compatible with many functional groups as demonstrated here by the use of unprotected peptides. Furthermore, aniline is soluble in a range of solvents and can be used as a convenient aqueous buffer with acetic acid, and both its solubility and nucleophilicity can be tuned through the introduction of substituents on the aromatic ring. Aniline catalysis will have a major impact on the numerous applications of imine chemistry including bioconjugation,[1–8] cellular labeling,[18,19] surface immobilization,[20] and dynamic combinatorial libraries.[21,22]

Received: July 19, 2006
Published online: October 19, 2006

Keywords: bioconjugation · nucleophilic catalysis · peptides · Schiff bases · transimination

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