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Enhanced Catalysis of Oxime-Based Bioconjugations by Substituted Anilines

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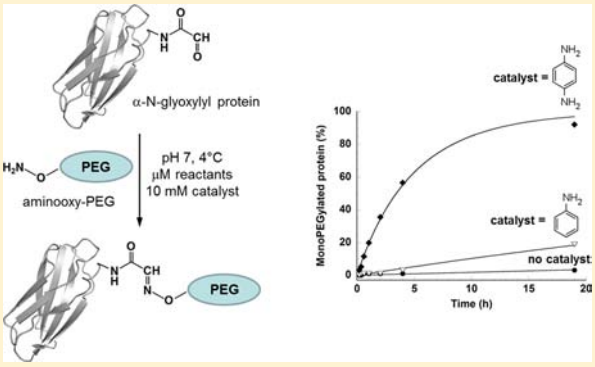
Supporting Information

ABSTRACT: The conjugation of biomolecules by chemoselective oxime ligation is of great interest for the site-specific modification of proteins, peptides, nucleic acids, and carbohydrates. These conjugations proceed optimally at a reaction pH of 4−5, but some biomolecules are not soluble or stable under these conditions. Aniline can be used as a nucleophilic catalyst to enhance the rate of oxime formation, but even in its presence, the reaction rate at neutral pH can be slower than desired, particularly at low reagent concentrations and/or temperature. Recently, alternative catalysts with improved properties were reported, including anthranilic acid derivatives for small molecule ligations, as well as *m*-phenylenediamine at high concentrations for protein conjugations. Here, we report that *p*-substituted anilines containing an electron-donating ring substituent are superior catalysts of oxime-based conjugations at pH 7. One such catalyst, *p*-phenylenediamine, was studied in greater detail. This catalyst was highly effective at neutral pH, even at the low concentration of 2 mM. In a model oxime ligation using aminooxy-functionalized PEG, catalysis at pH 7 resulted in a 120-fold faster rate of protein PEGylation as compared to an uncatalyzed reaction, and 19-fold faster than the equivalent aniline-catalyzed reaction. *p*-Phenylenediamine (10 mM) was also an effective catalyst under acidic conditions and was more efficient than aniline throughout the pH range 4−7. This catalyst allows efficient oxime bioconjugations to proceed under mild conditions and low micromolar concentrations, as demonstrated by the PEGylation of a small protein.



INTRODUCTION

Imine-based reactions are gaining increasing interest for the conjugation of complex biomolecules. Imines formed from ligation of α-effect amines with carbonyl groups, specifically those yielding hydrazone and oxime linkages, have found the greatest use thanks to the stability of these bonds and compatibility with other functional groups found in biomolecules. Research applications have included the total synthesis of peptides and proteins,[1−6] the modification of cell surfaces,[7−9] the introduction of protein labels *in vitro* and *in vivo*,[10−12] and the functionalization of solid surfaces[13] and nanoparticles.[14] There is also considerable interest in applying this chemistry to the development of site-specifically modified therapeutic conjugates, such as proteins modified with poly(ethylene glycol) (PEG) for half-life extension,[15−18] or tumor-directed antibodies coupled to cytotoxic payloads.[19,20]

Aldehydes and ketones can be introduced into proteins by a variety of means,[3,10,12,16,21−25] and reaction of these with hydrazide or aminooxy-derivatized substances leads to hydrazone and oxime conjugates. As the oxime linkage is hydrolytically more stable than a hydrazone,[26] this is a preferred imine for preparation of biotherapeutic conjugates, but formation of oximes is typically slower than that of hydrazones. Oxime ligations proceed with modest reaction rates under acidic conditions but can be extremely slow at neutral pH with $t_{1/2}$ in the order of several days. To improve the reaction rates, oxime conjugations ideally require millimolar concentrations of each reactant, or a large excess of one component. The requirement of low pH and high concentration presents a challenge to the use of this chemistry for bioconjugations. Many biomolecules are sensitive to low pH conditions, exhibit low solubility, or may be in limited supply.

An important development in the application of hydrazone and oxime conjugations to biomolecules came about with the introduction of aniline as a reaction catalyst.[27,28] In the case of oxime ligations, aniline can enhance the rates of formation by 1−3 orders of magnitude depending on experimental conditions. Enhancement of reaction rates by aniline can be explained by nucleophilic catalysis.[29] This involves the formation of a protonated aniline Schiff base as an intermediate

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that subsequently functions as an electrophile in a transimination reaction. Importantly, the intermediate aniline Schiff base must have a smaller equilibrium constant than the final product in order to ensure that the reactive intermediate does not compete with the formation of the desired product. Over the past few years, aniline has been used to catalyze the formation of a variety of hydrazone and oxime-based bioconjugates.[9,11,12,24,25,30−34]

The pK_a of aniline is 4.6, and that of the Schiff base formed with an aminooxy group is ∼2 units below this.[35] Consequently, aniline is most effective as a catalyst at acidic pH, but functions less well at neutral pH as the aniline Schiff base must be protonated to undergo transimination. Aniline derivatives with an elevated pK_a could be improved catalysts at neutral pH as the corresponding Schiff base intermediate might also exhibit a higher pK_a value. To this end, Dirksen et al. suggested the use of aniline derivatives with higher pH and described the use of *p*-methoxyaniline (pK_a = 5.3) to catalyze an oxime ligation at pH 7.0,[27] but its effect was not compared directly to that of aniline. In a study of carbohydrate oxime ligations, Thygesen et al.[30] found that *p*-methoxyaniline was a better catalyst than aniline at pH 7, but not at pH 4.5 where aniline was superior.

Recently, anthranilic acid derivatives and aminobenzoic acids were described as improved catalysts for small molecule hydrazone and oxime ligations, though their effect on protein conjugations was not evaluated.[36] Similarly, *m*-phenylenediamine emerged from a screen of aniline derivatives as a nucleophilic catalyst of oxime ligations; however, it was only moderately more effective than aniline and its primary advantage was enhanced solubility enabling its use at much higher concentrations of up to 750 mM.[37]

In an effort to identify improved catalysts for oxime-based bioconjugations at neutral pH and low reactant concentrations, we investigated a series of aniline derivatives for their potential to catalyze the conjugation of a model therapeutic protein with PEG. This covalent modification is of great significance as a well established approach to increase the half-life of biotherapeutics *in vivo*, reduce immunogenicity, improve solubility, and reduce the proteins' susceptibility to proteolytic degradation.[38] Biologically expressed proteins with N-terminal Ser/Thr residues can be selectively modified at their N-terminus to yield a glyoxylyl aldehyde group, an approach that has been widely utilized for site selective labeling because it requires little optimization or the use of noncoded amino acids. Here we have focused on the oximation of this broadly useful protein−aldehyde which yields a hydrolytically stable bond at the conjugation site. Included in this study are the analogues recently described for catalysis of small molecule oxime ligations[36] to evaluate their broader scope for bioorthogonal ligations of larger molecules at low (micromolar) reactant concentrations. We describe various aniline derivatives that can significantly accelerate the formation of oxime linkages, and are superior to aniline at pH 7. Moreover, we find that *p*-phenylenediamine is a superior catalyst throughout the pH range of 4−7.

■ EXPERIMENTAL SECTION

Reagents. Recombinant model protein T3 is a nontarget binding example of a Tn3 scaffold protein, an engineered protein module derived from the third fibronectin type III domain of human tenascin C.[39] T3 contained a single disulfide bond, and had the following amino acid sequence:

SQIEVKDVTDTTALITWFKPLAEIDGCELTY-
GIKDVPGDRTTIDLTEDENQYSIGNLKPDTEYEV-
SLICRRGDMSSNPAKETFTTGLGGGGHHHHHHHH

T3 was expressed in *E. coli* BL21 and purified by IMAC and subsequent anion exchange chromatography on Q FF sepharose. Aminooxy-functionalized PEG-20, i.e., methoxy-PEG-CONH$(CH_2)_2$−ONH_2 was obtained from NOF America (20 kDa, Sunbright ME-200CA). Aniline; *o*-, *m*-, and *p*-phenylenediamine; 2-methyl-*p*-phenylenediamine dihydrochloride; *o*-, *m*-, and *p*-aminophenol; *p*-methoxyaniline; 3,5-diaminobenzoic acid dihydrochloride; hydroxylamine hydrochloride; DMSO; sodium periodate; ethylene glycol; and all buffer salts were obtained from Sigma. *N*-Methyl-*p*-phenylenediamine dihydrochloride was from Acros Organics, 2-amino-5-methoxybenzoic acid from Alfa Aesar and L-serine from J. T. Baker.

Periodate Oxidation of N-Terminal Serine. Periodate oxidation was performed either in 1% ammonium bicarbonate (pH 7.7) or in 25 mM sodium phosphate, 140 mM NaCl, pH 7.4. The protein was transferred into the respective buffer by dialysis or by diafiltration. Sodium periodate solution was prepared fresh as a 91 mM stock solution in water. Final reactant concentration during the oxidation was 2 mg/mL (182 μM) for the protein and 910 μM sodium periodate, resulting in a 5-fold molar periodate excess. After periodate addition, the solution was allowed to incubate for 10 min at room temperature. Oxidation was terminated either by the addition of a 2000-fold molar excess of ethylene glycol over periodate or by the addition of serine to a final concentration of 5 mM. After addition of the quencher, the solution was mixed and incubated for 10 min at room temperature, before transferring samples by dialysis into the desired reaction buffer for oxime ligation (100 mM sodium phosphate or citrate at selected pH values).

Protein oxidation was near quantitative under these conditions, as confirmed by ESI mass spectrometry (Figure S1). Spectra showed the presence of two species, with $[M+H]^+$ values corresponding to the unhydrated and hydrated form of the expected α-*N*-glyoxylyl derivative, in addition to a low level of residual unoxidized material. For quantitative data analysis, a 95% conversion into the oxidized species was assumed.

Oxime Ligations. For conjugation of PEG to protein T3 (PEGylation), the reactions were performed at a protein concentration of 1 mg/mL (91 μM), and an aminooxy-PEG concentration of 455 μM (i.e., 5:1 PEG:protein molar ratio). Reactions at pH 6 and 7 were performed in 100 mM sodium phosphate buffer, while 100 mM sodium citrate was used for reactions at pH 4 and 5. Catalysts were initially dissolved as a stock solution of 0.6 or 1 M in either buffer (*N*-methyl-*p*-phenylenediamine dihydrochloride and 2-methyl-*p*-phenylenediamine dihydrochloride) or DMSO and then added to the reaction mixture to a final concentration of 10 mM (<2% v/v final DMSO where applicable). Reactions were allowed to proceed at either 4 °C or ambient temperature with protection from light and with mild agitation in 96 deep-well plates. Quenching was performed after selected time points by the addition of hydroxylamine hydrochloride to a final concentration of 18.2 mM.

Ligation reactions were analyzed by anion exchange HPLC, using a POROS HQ/10 (4.6 × 50) column (Applied Biosystems) with 20 mM Tris, pH 8.0, as equilibration buffer and 20 mM Tris, 0.5 M NaCl (pH 8.0) as elution buffer on an Agilent HPLC system. Elution was performed with a gradient from 0% to 100% B over 5 column volumes at a flow rate of 2

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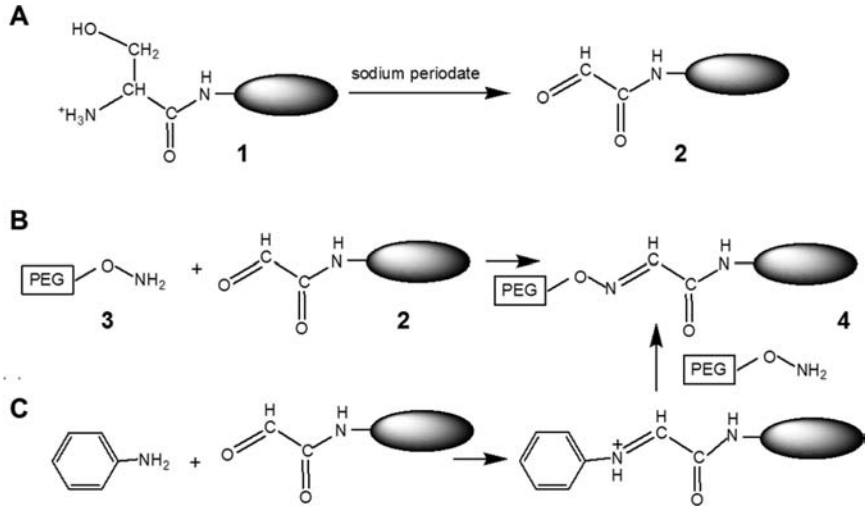

Figure 1. Reaction scheme: (A) Introduction of aldehyde functionality by periodate oxidation of N-terminal serine (or threonine) residues. (B) Oxime ligation by reaction with an aminooxy-functionalized PEG. (C) Nucleophilic catalysis by aniline (and derivatives) proceeds via a protonated Schiff base that undergoes transimination to the oxime end product.

mL/min. The identity of the peak corresponding to the PEGylated protein was confirmed by SDS-PAGE analysis. The reaction yield was quantitated by integration of the reactant and product peaks in the 280 nm chromatographic trace. For calculation of reaction kinetics, data were fitted to the second-order rate equation, assuming negligible reversibility of the oxime, as described in the Supporting Information.

Single-Pot Oxidation/Oxime Ligation. To allow protein oxidation and subsequent oxime coupling without intermediate buffer exchange, reaction conditions were modified as follows: T3 protein at a concentration of 10, 100, or 600 μM was reacted with an equimolar concentration of sodium periodate in 105 mM sodium phosphate, 125 mM NaCl, pH 7.0. Reactions were allowed to proceed for 15 min at room temperature with mild agitation, and any unreacted periodate was then quenched by the addition of serine to a final concentration of 6 mM. After addition of serine, the mixture was allowed to stand for 1 h at room temperature. Subsequent oxime coupling was performed in the same reaction mixture by addition of aminooxy-PEG-20 stock solutions in 100 mM sodium phosphate, pH 7.0. A total of six reactions were set up containing oxidized T3 protein at final concentrations of 9, 90, or 200 μM, together with 2 or 5 mol equiv of aminooxy-PEG. *p*-Phenylenediamine was added as catalyst to a final concentration of 10 mM. Reaction tubes were covered with foil and incubated for 22 h at ambient temperation. Reactions were analyzed as above, but without hydroxylamine hydrochloride quenching.

RESULTS

Substituted Anilines as Catalysts of Oxime Ligation. To compare the effectiveness of different catalysts in promoting oxime-based bioconjugations at neutral pH, we measured ligation rates for the reaction of a small model protein, designated T3, containing an N-terminal aldehyde group. As a first step, an aldehyde function was introduced by mild periodate oxidation of the N-terminal serine residue in protein T3 (Figure 1A). To simulate a conjugation reaction that is relevant to biotechnology, we studied the reaction of aminooxy-poly(ethylene glycol) (PEG) with T3 protein. As shown in Figure 1B, the resulting α-*N*-glyoxylyl-group of T3 can site-specifically react with aminooxy-PEG, forming a stable oxime linkage. If aniline (or a derivative) is present as a nucleophilic catalyst, the reaction proceeds via an intermediate protonated aniline Schiff base that subsequently acts as an electrophile in a transimination reaction (Figure 1C). The following practical constraints were placed on the PEGylation reaction: (i) a protein concentration of less than 0.1 mM was used to mimic the limited solubility or availability that can occur with larger macromolecules; (ii) a high molecular weight PEG derivative (20 kDa) was used, as is typical for monoPEGylation of protein therapeutics. In addition, a modest catalyst concentration (10 mM) was used to extend the relevance of this work to oxime ligations involving living cells or conformationally sensitive proteins, two applications where higher concentrations of catalyst can be problematic.[9,32] Combined with reaction at neutral pH, these constraints were expected to diminish oxime formation rates and therefore help distinguish the efficacy of different reaction catalysts.

A panel of aniline derivatives was selected to assess their potential for enhancing the rate of aminooxy-PEG conjugation to T3 protein (Figure 2). Substituted anilines with amino, hydroxy, or methoxy substituents were chosen, as the pK_a for several of these is higher than that of unsubstituted aniline. This panel also included two aminobenzoic acid derivatives that were recently described as catalysts for oxime and hydrazone ligations.[36] Conjugations were carried out at pH 7 and ambient temperature, using 1 mg/mL oxidized T3 protein (91 μM), 5 equiv (455 μM) of aminooxy-PEG, 10 mM aniline, or derivative. Samples from each reaction were taken after 4 h and 20 h and quenched by addition of excess hydroxylamine. The yield of PEG conjugate was determined by analytical anion exchange chromatography, using conditions which readily resolve the conjugate from unmodified protein (see Figure S2).

Under the reaction conditions used, PEG conjugation proceeded very poorly without catalyst and would have needed weeks to reach equilibrium (Figure 3A). Unsubstituted aniline had a moderate catalytic effect while the substituted anilines had a spectrum of activities ranging from inhibition to significant acceleration of oxime product formation. Surprisingly, the strongest effect on activity was the position of the electron donating group on the aniline ring. For instance, *p*-substituted aminophenol and phenylenediamine were substan-

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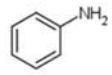
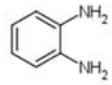
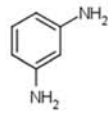
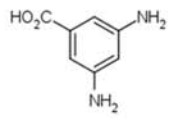
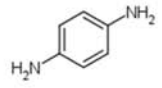
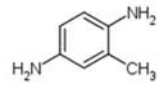
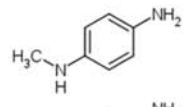
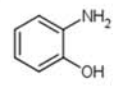
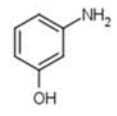
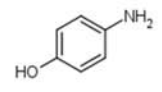
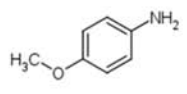
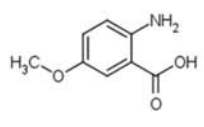
Catalyst	Structure	Abbreviation	pK_a
aniline		An	4.62
o-phenylenediamine		*o*-PDA	4.47
m-phenylenediamine		*m*-PDA	4.88
3,5-diaminobenzoic acid		3,5-DABA	
p-phenylenediamine		*p*-PDA	6.08
2-methyl-*p*-phenylenediamine		2-Me-*p*-PDA	
N-methyl-*p*-phenylenediamine		*N*-Me-*p*-PDA	
o-aminophenol		*o*-AP	4.72
m-aminophenol		*m*-AP	4.17
p-aminophenol		*p*-AP	5.50
p-methoxyaniline		*p*-MeOAn	5.29
5-methoxy-anthranilic acid		5-MeO-AA	

Figure 2. Aniline derivatives evaluated as catalysts for oxime ligation. Structures of compounds studied here. Values for pK_a are from *Jencks & Regenstein*.[41] For compounds with more than one amine pK_a, only the highest one is given. pK_a values are not available for 3,5-DABA, 2-Me-*p*-PDA, *N*-Me-*p*-PDA, and 5-MeO-AA, but might be expected to be higher than that of *m*-PDA, *p*-PDA, *p*-PDA, and *p*-MeOAn, respectively, due to the elevated pK_a's of anthranilic acid (4.80), *m*-toluidine (4.67), and *N*-methylaniline (4.85) versus aniline.

tially better catalysts than unsubstituted aniline, but the *o*-isomers were less effective, and the *m*-isomers significantly inhibited the rate of reaction. Under the conditions employed in our study, *m*-phenylenediamine, described by Rashidian et al.[37] as a catalyst of oxime formation, did not enhance the reaction rate, but inhibited the formation of product as compared to the uncatalyzed ligation. In agreement with the work of Crisalli and Kool,[36] we did find that 5-methoxyanthranilic acid was an efficient catalyst for the protein conjugation studied here, while 3,5-diaminobenzoic acid, also described in that same work, was only moderately effective. To evaluate the effect of *m*-phenylenediamine at a higher concentration, we repeated the *m*-PDA experiment at a concentration of 200 mM (having first titrated a 1 M *m*-PDA stock solution to ensure it was buffered to pH 7.0). Under these conditions we observed <1% oxime product formation after 12 h, compared to 7% product for an uncatalyzed control reaction. Thus, higher concentrations of *m*-phenylenediamine, as used in Rashidian et al.,[37] did not alleviate the inhibitory effect on oxime product formation.

To further compare unsubstituted and *p*-substituted anilines, the time course of conjugation was assessed under the same reaction conditions. These data further confirmed the moderate catalytic effect of unsubstituted aniline (10 mM) at pH 7, and much superior activity for this set of *p*-substituted anilines (Figure 3B). While aniline only achieved a 2.7-fold enhancement in the oxime ligation rate, catalysis by the substituted anilines resulted in up to 67-fold faster reaction rates (Table

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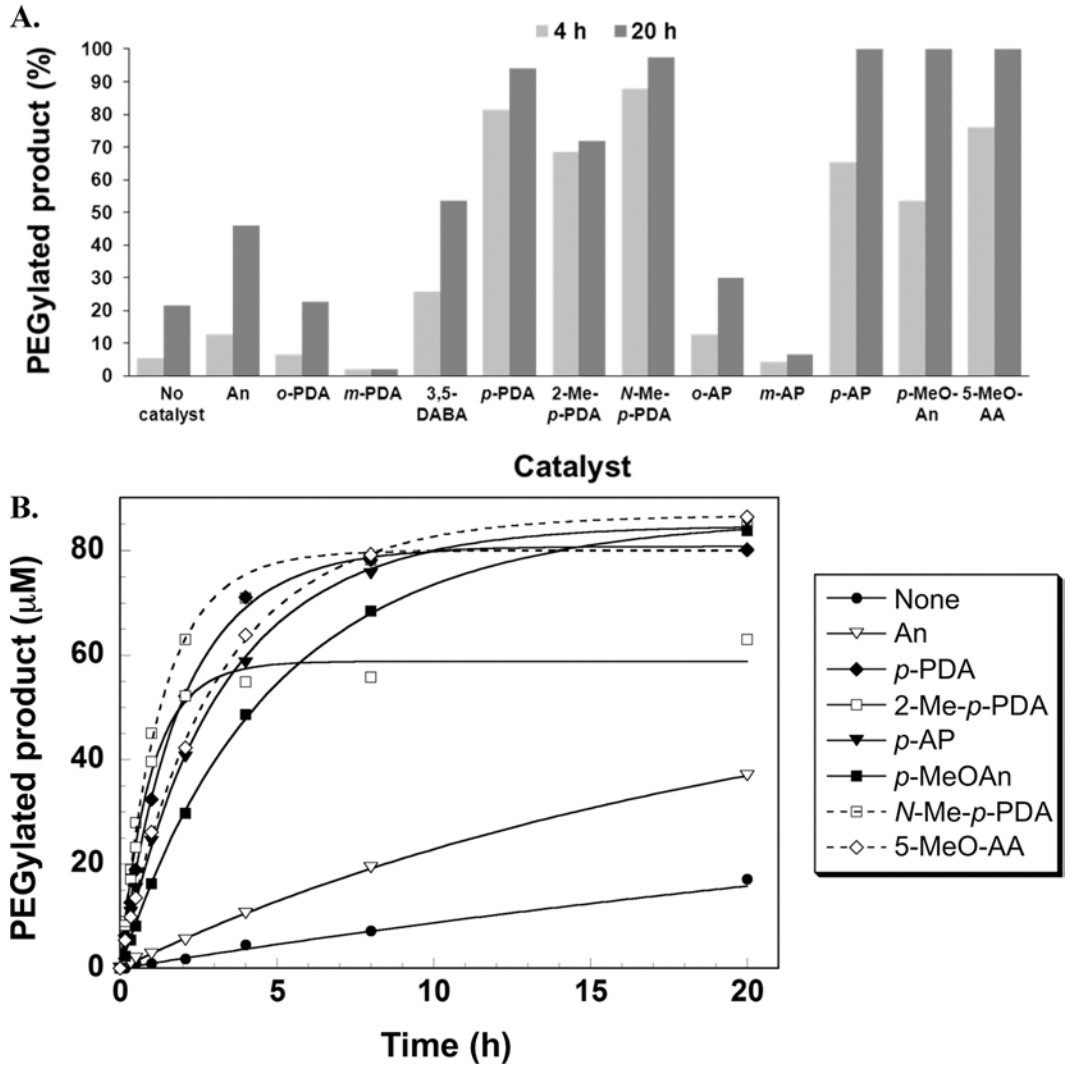

Figure 3. Catalysis of oxime ligation by aniline derivatives. A. Formation of oxime ligation product at pH 7 in the presence of aniline derivatives as nucleophilic catalysts. The reaction between oxidized T3 protein and aminooxy-PEG (20 kDa) was performed at a protein concentration of 1 mg/mL (91 μM) and a 5-fold molar excess of PEG (455 μM) in 100 mM sodium phosphate, pH 7.0, in the presence of 10 mM of the respective aniline derivative. Reactions were allowed to proceed at room temperature for 4 or 20 h prior to quenching and analysis by HP IEC. B. Formation of oxime ligation product over time at pH 7 in the absence of catalyst or in the presence of aniline and derivatives. The reaction was performed at a protein concentration of 1 mg/mL (91 μM) and a PEG concentration of 455 μM at room temperature. Product formation was analyzed by HP IEC, and data were fitted to the second order rate equation.

S1). The rank order of activity, with respect to substituent, was amino > hydroxy > methoxy, which also correlated with decreasing pK_a for each of the singly protonated anilinium species. Under these conditions, the *o*-carboxylate group in 5-methoxyanthranilic acid resulted in a minor catalytic enhancement (less than 2-fold) as compared to the structurally related *p*-methoxyaniline. During these studies, we observed that the *p*-aminoanilines in this group, as well as *p*-aminophenol, were prone to formation of colored oxidation products under experimental conditions that did not involve an inert gas atmosphere. Reactions in the presence of *N*-methyl-*p*-phenylenediamine and 2-methyl-*p*-phenylenediamine were the quickest to become colored and also formed a brown precipitate after several hours of incubation, likely due to the formation of azobenzene/quinone species. Due to the high catalytic efficiency observed for *p*-phenylenediamine and greater oxidative stability (Figure S3), we chose to study this catalyst in greater detail.

***p*-Phenylenediamine is a Superior Catalyst Across a Broad pH Range.** Each of the aniline derivatives with enhanced neutral pH activity had a higher pK_a than unsubstituted aniline. Under acidic conditions, aniline may be a better catalyst of oxime formation, as protonation of the catalyst will reduce the rate of intermediate Schiff base formation. Phenylenediamines are unique in having two amines available for Schiff base formation, with pK_a's that are several units apart (pK_a 6.08 and 3.29 for *p*-phenylenediamine). For this reason, we examined the catalytic activity of *p*-phenylenediamine across this pH range.

Using the same reagent and catalyst concentrations as in earlier studies, the rate of oxime formation between aminooxy-PEG and glyoxylyl-derivatized T3 protein was assessed as a function of reaction pH, either uncatalyzed or in the presence of aniline or *p*-phenylenediamine. We also compared reaction rates at 4 °C to ambient temperature. Under all conditions studied, *p*-phenylenediamine showed superior catalytic activity

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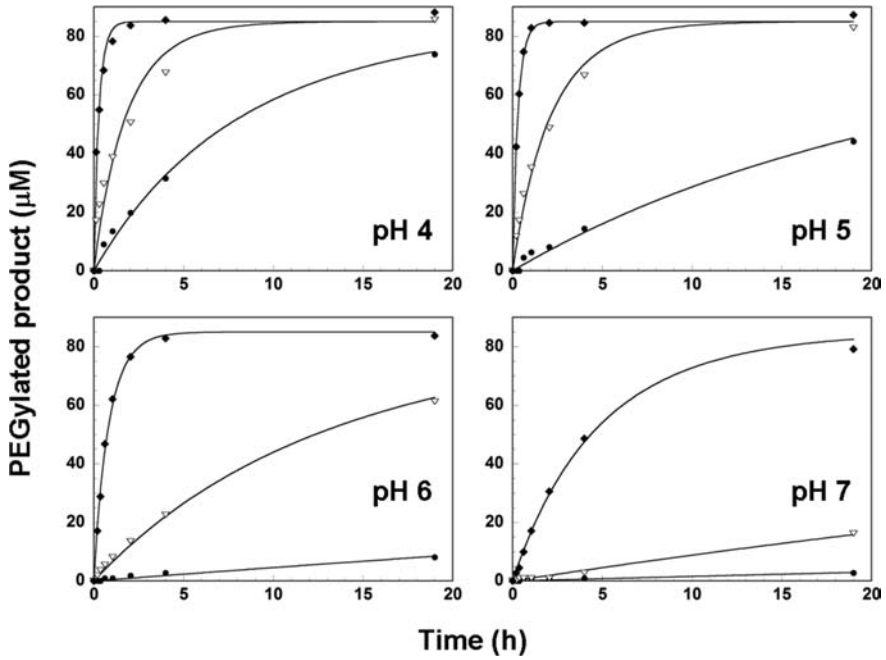

Figure 4. Rate of formation of oxime ligated T3-PEG product at 4 °C as a function of catalyst and pH. Reactions were performed with a protein concentration of 1 mg/mL (91 μM) and a PEG concentration of 455 μM in the absence of catalyst (filled circles) or in the presence of aniline (empty triangles) or *p*-phenylenediamine (filled diamonds).

Table 1. Rates of Oxime Ligation as a Function of pH, Catalyst, and Temperature[a]

		k_{obs} (10^{-3} M^{-1} s^{-1})				
pH	temp.	no catalyst	An	*p*-PDA	$k_{obs(p\text{-PDA})}/k_{obs(uncat)}$	$k_{obs(p\text{-PDA})}/k_{obs(An)}$
4	4 °C	83 ± 4.9	380 ± 52	2600 ± 210	31	6.8
	RT	220 ± 17	1200 ± 77	4700 ± 110	21	3.9
5	4 °C	28 ± 1.4	320 ± 21	2400 ± 54	85	7.5
	RT	88 ± 5.1	810 ± 11	4300 ± 260	49	5.3
6	4 °C	3.6 ± 0.24	51 ± 1.7	850 ± 26.0	240	17
	RT	14 ± 0.20	140 ± 1.9	1500 ± 68	110	11
7	4 °C	1.2 ± 0.20	7.2 ± 0.30	140 ± 5.4	120	19
	RT	6.0 ± 0.12	17.0 ± 0.30	290 ± 29	48	17

[a]Calculation of second order rate constants (k_{obs}) for formation of T3-PEG conjugate by oxime ligation, in the presence or absence of catalyst. Reactions were carried out using 1 mg/mL oxidized T3 protein (91 μM), 455 μM aminooxy-PEG, and 10 mM catalyst where noted. Columns to the right show the rate enhancement of *p*-phenylenediamine-catalyzed reactions relative to uncatalyzed or aniline-catalyzed samples. Abbreviations: RT = room temperature; An = aniline; *p*-PDA = *p*-phenylenediamine; uncat = uncatalyzed.

compared to aniline. Figure 4 and Figure S4 illustrate the time course of the reaction at 4 °C and at room temperature, respectively. Second order rate constants for oxime product formation were computed by fitting data to the appropriate rate equation (Table 1). As expected, aniline enhanced the rate of oxime formation at all pH values and at both temperatures studied. Under the conditions used here, aniline accelerated the rate of oxime PEGylation from 2.8- to 14-fold relative to the uncatalyzed reaction. By contrast, rate enhancements by *p*-phenylenediamine were in the range of 21- to 240-fold. Relative to the aniline-catalyzed reaction, reaction rates with *p*-phenylenediamine were 3.9- to 19-fold faster, with the greatest difference occurring at higher pH values.

We also compared the effect of catalyst concentration on rates of oxime formation at pH 6 and 7, by again performing a time course experiment, but this time in the presence of 0, 2, or 10 mM catalyst. Strikingly, even at the low concentration of 2 mM, *p*-phenylenediamine allows the reactions to proceed rapidly at both pH values and at both temperatures studied, with only moderate reduction in rate enhancement compared to a 10 mM catalyst concentration (Table 2).

***p*-Phenylenediamine Catalysis Facilitates One-Pot *α*-*N*-Glyoxylyl Formation and Oxime Ligation.** Selective periodate oxidation of N-terminal serine or threonine residues is optimally performed at pH 7–8.[21] The ability to perform efficient oxime ligations at neutral pH should alleviate the need for a buffer exchange between oxidation and conjugation steps for all applications where aldehydes are introduced into proteins by periodate oxidation. We investigated the feasibility of a simplified one-pot strategy as detailed in experimental procedures, that allows the 2-step process of protein oxidation and oxime ligation to proceed without the need for intermediate purification. As shown in Figure 5, this approach is perfectly feasible, allowing convenient biomolecular ligations with very satisfactory yield. Although the yield was found to be slightly lower compared to the procedure that incorporates a buffer exchange after oxidation, the convenience of this approach makes *p*-phenylenediamine-catalyzed reactions partic-

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Table 2. Rates of Oxime Ligation As a Function of Catalyst Concentration[a]

pH	temp	catalyst conc. (mM)	k_{obs} (10^{-3} M^{-1} s^{-1}) no catalyst	An	*p*-PDA
6	4 °C	-	2.9 ± 0.36		
		2		17 ± 1.3	410 ± 18
		10		44 ± 4.3	980 ± 23
	RT	-	15 ± 0.39		
		2		53 ± 1.7	960 ± 16
		10		140 ± 3.8	1900 ± 49
7	4 °C	-	1.2 ± 0.24		
		2		2.4 ± 0.26	95 ± 8.7
		10		5.0 ± 0.42	140 ± 14
	RT	-	8.8 ± 0.15		
		2		13 ± 0.24	190 ± 22
		10		20 ± 0.28	290 ± 18

[a]Calculation of second order rate constants (k_{obs}) for formation of T3-PEG conjugate in the presence of different catalyst concentrations. Reactions were carried out using 1 mg/mL oxidized T3 protein (91 μM) and 455 μM aminooxy-PEG. Abbreviations: RT = room temperature; An = aniline; *p*-PDA = *p*-phenylenediamine.

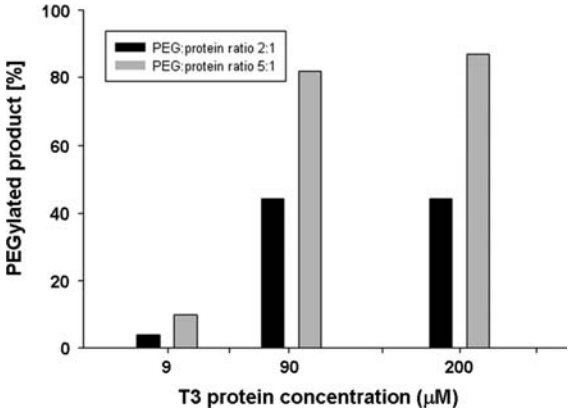

Figure 5. Formation of oxime PEGylated product for different protein concentrations when periodate oxidation and oxime coupling are performed in a sequential one-pot experimental setup. Black bars represent product yields for reactions containing 2 mol equiv of aminooxy-PEG (i.e., 18, 180, and 400 μM). Gray bars represent product yields for reactions containing 5 mol equiv of aminooxy-PEG (i.e., 45, 450, and 1000 μM). All reactions contained 10 mM *p*-PDA catalyst, and were incubated for 22 h at ambient temperature prior to analysis.

ularly attractive for larger scale applications in development and manufacturing.

DISCUSSION

Since its description in 2006 as a catalyst of oxime ligation,[27] aniline has been widely used to promote a variety of imine-based conjugations.[9,11,12,24,25,30−34] In most of these studies, conjugations have been carried out at the optimal reaction pH of 4−5, where the catalyzed (and uncatalyzed) reaction rates are maximal. While many biomolecules are tolerant of this level of acidity, others are not stable or soluble under these conditions. This necessitates ligation reactions at or near neutral pH, where the rate of aniline-catalyzed oxime formation may be slower than that of the uncatalyzed reaction at pH 4 (for example, see Table 1). In addition to pH sensitivity, some proteins may also be unstable in the presence of the high aniline concentrations (100 mM) needed to achieve adequate oxime ligation rates.[32] The need for a mild reaction pH and modest catalyst concentration is also true of oxime-labeling applications with living cells.[9,11] Finally, the catalytic effect of aniline can vary considerably depending on the reaction conditions, ligation groups, and aniline concentration, so that some researchers have found no advantage to using this catalyst with highly activated aldehydes.[40] For these reasons, improved catalysts of oxime ligations could be broadly useful.

In this work, we have identified substituted anilines that are superior catalysts for oxime-based bioconjugation, and enable these reactions to proceed under mild conditions at neutral pH. The common feature of each of these catalysts was an electron donating amino, hydroxy, or methoxy group at the *para* position of the aniline aromatic ring. In principle, *para* substitution best combines the ability to electronically tune the amino nucleophile compared to *meta* substitution and without the steric effects often seen upon *ortho* substitution. Within this group of *p*-substituted anilines, catalytic activity was also correlated with the pK_a of the anilinium conjugate acid. Although we only tested −OH, −OR, −NH_2, and −NHR substituted anilines, other electron donating *para* substitutions, such as in *p*-alkyl anilines, may also show improved catalytic activity at neutral pH.

The difference in reactivities for positional isomers of aminophenol and phenylenediamine was particularly surprising. While the *para*-isomers of these compounds were efficient catalysts, the *ortho*- and *meta*-isomers were much less effective, or even inhibited the rate of oxime ligation. In contrast to previous reports that described *m*-phenylenediamine as an efficient catalyst for oxime-based bioconjugations at high concentrations,[37] we found that this compound had a strong inhibitory effect on the reaction studied here. Similarly, we found that *m*-aminophenol also inhibited the oxime-mediated conjugation of PEG. We are unsure of the mechanistic reasons for these distinct observations, but the key differences in our study were the use of macromolecular substrates, a glyoxylyl group as the reactive aldehyde function, and the use of lower catalyst concentrations. We ruled out the catalyst concentration as a factor, as formation of the T3-PEG oxime conjugate was also inhibited in the presence of 200 mM *m*-phenylenediamine. Inhibition by *m*-phenylenediamine may therefore be specific to the glyoxylyl functionality, but our inability to readily incorporate alternative aldehyde or ketone groups into T3 protein made it impossible to confirm this. In any case, the reported benefit of *m*-phenylenediamine over aniline was due to its superior solubility limit at pH 7, allowing reactions to be carried out at very high catalyst concentrations.[37] However, as noted previously, high concentrations of organic catalysts may be problematic for bioconjugations involving conformationally sensitive proteins[32] or living cells.[9,11] High catalyst concentrations can also create practical difficulties with buffering of solutions or disposal of organic waste.

In this work, we found that catalysis of oxime ligation by *p*-phenylenediamine was superior to aniline throughout the pH range of 4−7. This may be the result of phenylenediamines having a second amine group that remains mostly unprotonated at acidic pH (*p*-phenylenediamine pK_{a2} = 3.29). The formation of Schiff bases involves two steps, initial attack by an amine on the carbonyl function leading to a carbinolamine intermediate, followed by acid-catalyzed dehydration to yield the Schiff base species. For aniline Schiff base formation, the rate determining

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step at neutral pH is dehydration of the carbinolamine addition product, but at acidic pH, amine attack becomes rate limiting due to aniline protonation.[35] Thus, the enhanced catalytic activity of *p*-phenylenediamine over aniline under neutral and acidic reaction conditions may result from having amino groups that remain unprotonated throughout this pH range. Consistent with the work of Crisalli and Kool,[36,42] we found that 5-methoxyanthranilic acid was also an efficient catalyst of the oxime-based protein conjugation studied here. However, the rate enhancement relative to the aniline-catalyzed reaction was not considerably more pronounced than that observed for *p*-methoxyaniline, suggesting that the *p*-methoxy group is primarily responsible for the enhanced catalytic activity, with a more modest contribution from the *o*-carboxylate group.

In summary, we have shown that *p*-phenylenediamine is a highly efficient catalyst for oxime-based bioconjugation reactions that allows ligations to proceed under mild conditions (neutral pH) and low micromolar reactant concentrations with excellent yield. As is the case for aniline, *p*-phenylenediamine is a weak base and a moderate nucleophile that is not expected to react with amino acid side chains of peptides and proteins. The catalyst can be readily removed by chromatography or diafiltration. Nucleophilic catalysis with *p*-phenylenediamine should be widely applicable to the numerous reactions that involve imine formation, including bioconjugation of proteins and nucleotides, labeling of cells, and immobilization of ligands on solid surfaces. This catalyst allows the highly efficient conjugation of complex biomolecules under mild conditions and low concentrations. As such, it should also be commercially valuable for the production of site-specifically conjugated biotherapeutics, like PEGylated proteins and antibody−drug conjugates.

■ ASSOCIATED CONTENT

Supporting Information

Additional data includes methodology used for calculating reaction rates, reaction rates for different aniline analogues (Table S1), mass spectrometric analysis of proteins used (Figure S1), chromatography used to quantitate reaction products (Figure S2), redox behavior of select catalysts (Figure S3), and reaction rates for aniline- and *p*-phenylenediamine-catalyzed reactions at room temperature (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): All authors, except PED, are current employees of MedImmune, LLC.

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