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Catalysis of hydrazone and oxime peptide ligation by arginine

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Supporting Information Placeholder



ABSTRACT: Hydrazone ligation of peptides α -oxo aldehydes with peptide hydrazides is catalyzed by arginine. The catalysis is assisted by side-chain guanidinium group. Hydrazone ligation proceeds efficiently in phosphate buffer at neutral pH but is particularly powerful in bicarbonate/CO₂ buffer. In addition to acting as a catalyst, arginine prevents the aggregation of proteins during ligation. With its dual properties as nucleophilic catalyst and protein aggregation inhibitor, arginine hydrochloride is a useful addition to the hydrazone/oxime ligation toolbox.

Imine-based reactions leading to the formation of hydrazones or oximes (X = N or O respectively, Figure 1) are popular bio-conjugation techniques recognized for their ease of implementation, their high chemoselectivity in water and their compatibility with a wide range of substrates including polypeptides, nucleic acids and nanomaterials.^{1,2}

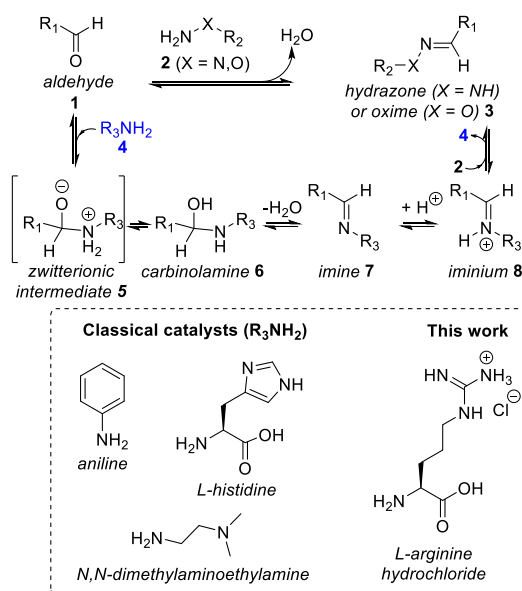


Figure 1. Principle of hydrazone or oxime ligations and of their nucleophilic catalysis by amine compounds. R₁, R₂ = Biomolecules, dyes, etc...

Hydrazone or oxime formation shows useful reaction rates at mildly acidic pH (4-5) but are very slow processes in water at

neutral pH, which is the preferred media for manipulating complex biomolecules such as proteins. As a consequence, many studies focused on accelerating these reactions by thermal activation,³ by addition of organic co-solvents³ or catalysts.¹ Nucleophilic catalysis by amine compounds such as aniline^{4,5} (or derivatives thereof⁶) or *N,N*-dimethylaminoethylamine^{7, 8} has proven especially powerful in this regard (Figure 1).

In such a process, the amine catalyst **4** combines with the aldehyde **1** to produce imine **7**, which undergoes a transimination with hydrazine or hydroxylamine derivative **2** through iminium species **8**. The action of *N,N*-dimethylaminoethylamine involves an intramolecular acid-catalysis by the protonated *N,N*-dimethylamino group which considerably accelerates the formation of the imine product of type **7** by facilitating water elimination from carbinolamine **6** (Figure 1).⁷ In an analogous way, histidine has been shown to catalyze hydrazone or oxime bond formation, likely by favoring the dehydration of carbinolamine **6** through intramolecular acid catalysis by the imidazolium side chain group.^{8,9}

Despite a great interest with regard to reagent cost, operational advantages, low toxicity and adherence to sustainable chemistry principles,¹⁰ only a few works have examined the catalysis of hydrazone or oxime ligations by proteinogenic amino acids. Hereinafter, we report that the amino acid arginine catalyzes hydrazone or oxime bond formation in phosphate buffer at neutral pH. Detailed kinetic studies suggest that the side-chain guanidinium group plays a crucial role in the catalysis mechanism. We also show that the rate of arginine-catalyzed hydrazone ligation is dramatically enhanced in bicarbonate/CO₂ buffer, a classical buffer used for cell culture.

The model hydrazone ligations used for investigating the catalytic properties of arginine, added in the reaction as arginine hydrochloride, and other additives are shown in Figure 2. The hydrazide and α -oxo aldehyde functionalities were chosen for being popular and easily installed on peptides or proteins through synthetic or semi-synthetic methods.^{11, 12}

In a first series of experiments, hydrazone ligation was performed with model peptides **8a** and **9a** in pH 7.0 sodium phosphate buffer at 25 °C at 1 mM peptide concentration and 50 mM concentration for the additives. The progress of the reaction was monitored by measuring the absorbance of the hydrazone product **10a** at 285 nm. Kinetic data were fitted to extract the apparent second order rate constants. The reaction mixtures were also analyzed by LC-MS. Figure 3a shows that arginine hydrochloride has a marked catalytic effect compared to alanine. The relationship between arginine concentration and the rate of hydrazone formation is remarkably linear, with a break at ~400 mM (Figure 3b). At this catalyst concentration, hydrazone ligation is a fast process with a k_{app} of ~10 M⁻¹ min⁻¹. Arginine hydrochloride was found to catalyze ketoxime bond formation as well at pH 6.0 or 7.0 (see Supplementary Information).

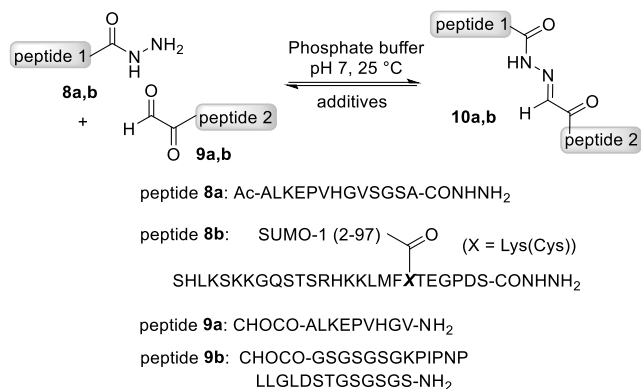


Figure 2. Model hydrazone ligations examined in this study.

The reaction between **8a** and **9a** was also catalyzed by histidine as reported for other types of aldehyde substrates.^{8,9} In this case however, the reaction yielded a significant amount of side-product in addition to the expected hydrazone. This side-product was identified as the α -oxo aldehyde/histidine condensate, thereby discarding the use of histidine as a suitable catalyst for hydrazone ligation with α -oxo aldehydes (see Supplementary Information).

At 50 mM catalyst concentration, we found that *N,N*-dimethylaminoethylamine (6.46 ± 0.05 M⁻¹.min⁻¹) performs better than arginine (2.71 ± 0.05 M⁻¹.min⁻¹). However, raising arginine concentration to 200 mM enables to achieve a k_{app} of 6 M⁻¹.min⁻¹ as well, with the advantage of using a catalyst of much lower toxicity. § Moreover, an interesting property of arginine hydrochloride is its well established capacity to inhibit protein aggregation.¹³ It is a popular additive for protein refolding experiments and is frequently used at ~0.5 M concentration or higher for that purpose. Having a catalyst of hydrazone or oxime ligation that inhibits protein aggregation is a strong asset when it comes to work with complex protein targets (vide infra).

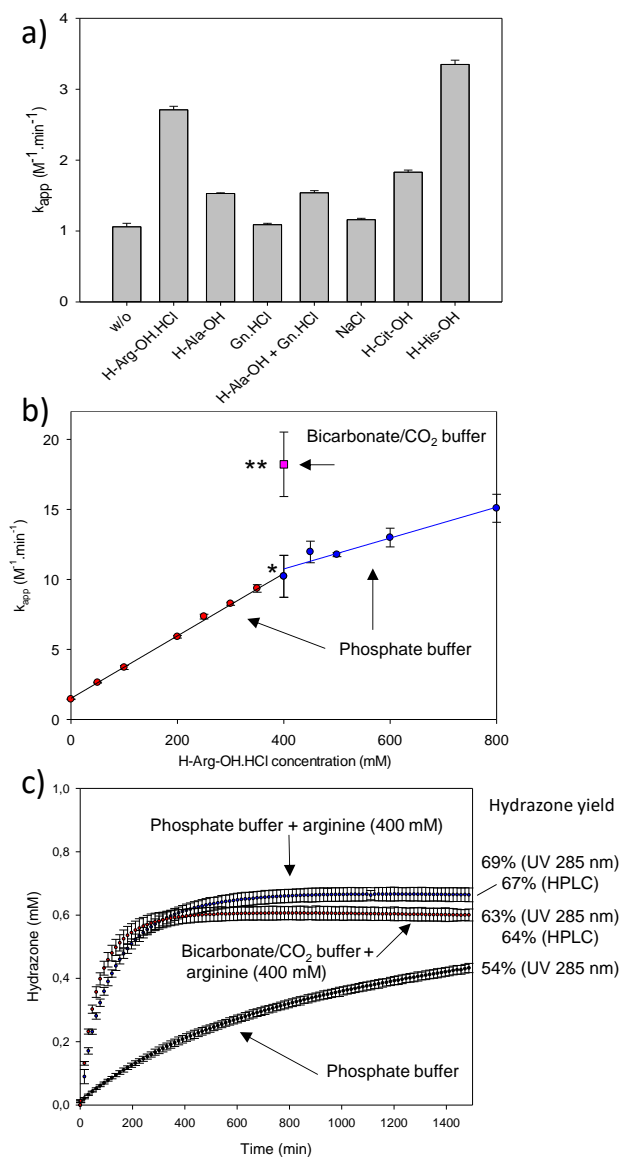


Figure 3. Apparent second order rate constant of hydrazone **10a** formation in the presence of amino acid and/or salt additives. Sodium phosphate buffer 0.1 M, ~1 mM final concentration for each peptide, pH ~7, 25 °C. The data correspond to the mean \pm standard error (95% confidence limit interval). a) The additive concentration was 50 mM. The reactions were performed in sextuplicate in one 96-well microtiter plate. The reaction with histidine is complicated by the formation of a covalent adduct between the COCHO peptide and histidine catalyst in significant amounts (see Supplementary Information). b) Effect of Arg.HCl concentration on the apparent second order rate constant of hydrazone formation in 0.1 M sodium phosphate (circles) or 40 mM bicarbonate/CO₂ buffer (square). * Mean of four independent experiments, each done in triplicate. ** Mean of five independent experiments, each done in triplicate. c) Time course of hydrazone **10a** formation from **8a** (0.95 mM) and **9a** (0.99 mM) at 25 °C in phosphate buffer pH 7.04, phosphate buffer + arginine (400 mM) pH 7.01 or bicarbonate/CO₂ buffer + arginine (400 mM) pH 7.07.

The data presented in Figure 3a show that sodium chloride, guanidine hydrochloride (Gn.HCl), or alanine plus Gn.HCl were all far below the catalytic level of arginine hydrochloride.

The same was observed for citrulline which has a neutral urea group in its side-chain in lieu of a guanidinium group as in arginine. These series of experiments suggest that the catalysis of the hydrazone ligation by arginine is based on an intramolecular catalytic participation of the side-chain guanidinium group. Contrary to tertiary ammonium or imidazolium cations, guanidinium cations are very weak acids including those found in arginine (pK_a 13.8).¹⁴ Logically, evidence for a role of arginine residue as an acid catalyst in enzymes are scarce.^{15, 16} The occurrence of a general acid-catalysis of the carbinolamine dehydration step (**6** \rightarrow **7** in Figure 1) by guanidinium cation, as observed for histidine or *N,N*-dimethylaminoethylamine, is therefore not obvious. Mechanisms other than general acid catalysis can impact imine formation. For example, some computational studies suggest that explicit water molecules can promote the dehydration of the carbinolamine **6** by facilitating an internal N-to-O proton shuffling mechanism.¹⁷ Interestingly, arginine is strongly suspected to act as a relay for proton transfers occurring in bacteriorhodopsin¹⁸ or pyridoxal 5'-phosphate synthase and other proteins.¹⁹⁻²¹ The question whether the side-chain guanidinium cation of arginine promotes hydrazone formation by facilitating proton transfers, by acting as a general acid catalyst or by another mean will require more investigations.

At high arginine hydrochloride concentrations (e. g. 400 mM), a few percent of a byproduct corresponding to the conversion of the α -oxo aldehyde moiety into a glycine residue was observed by LC-MS. We reasoned that the byproduct arose from a transamination of the α -oxo aldehyde peptide by the excess of added arginine, a reaction that is known to be promoted by trace metals. In accord with this hypothesis, byproduct formation could be suppressed by adding ethylenediaminetetraacetate (EDTA, 1 mM) to the ligation mixture (see Supplementary Information).

After having scrutinized the arginine-catalyzed hydrazone ligation using model peptides **8a** and **9a**, we examined the reaction of protein hydrazone **8b** (0.5 mM) with α -oxo aldehyde peptide **9b** (2.5 mM) in the presence of arginine hydrochloride (400 mM) in phosphate buffer (pH 7.0). Protein **8b** is a fully synthetic 14 kDa branched protein made of a SUMO-1 domain linked to a human p53 peptide. The sequence of peptide **9b** corresponds to V5 peptidic tag. The ligation was monitored by SDS-PAGE followed by Western-blot analysis (Figure 4). The anti-SUMO-1 antibody was used to visualize protein hydrazone **8b** and the hydrazone product **10b**, while anti-V5 antibody was used to reveal only the hydrazone product **10b**. These analyses show the successful formation of hydrazone product **10b** whose identity was confirmed by LC-MS and proteomic analysis (see Supplementary Information). The equilibrium was reached in about 300 min. The control experiment conducted in the absence of arginine catalyst failed to provide the expected protein hydrazone **10b** due to protein precipitation (see Supplementary Information). This example is a nice illustration of the dual properties of arginine which acts as a catalyst of hydrazone bond formation and as an inhibitor of protein aggregation.

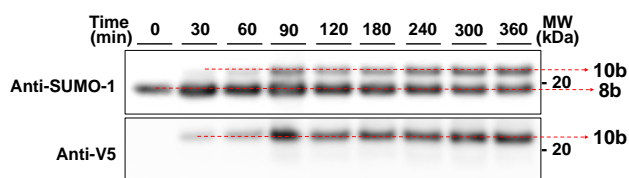


Figure 4. Hydrazone ligation between SUMO-1 p53 protein hydrazone **8b** and α -oxo aldehyde peptide **9b** yields protein hydrazone **10b**. Conditions: **8b** 0.5 mM, **9b** 2.5 mM, arginine hydrochloride 400 mM, 0.1 M sodium phosphate buffer pH 7.0, 25 °C.

After having validated the interest of arginine catalysis in phosphate buffer at the protein level, we were interested to know if catalysis proceeds also in bicarbonate/CO₂ buffer (pH 7.2-7.4), which is a classical buffering system used for cell culture media. Toward this end, an aqueous solution of sodium bicarbonate (40 mM) was equilibrated for two days in a standard CO₂ incubator for cell culture (5% pCO₂) and then used to dissolve arginine hydrochloride at 400 mM. With a resulting pH of ~7.0, the solution was used directly for performing the hydrazone ligation of peptides **8a** and **9a**. We found that the rate constant for the arginine-catalyzed reaction in bicarbonate/CO₂ buffer ($18.2 \pm 2.3 \text{ M}^{-1} \cdot \text{min}^{-1}$) was almost twice that measured in phosphate buffer at the same pH (Figure 3b, pink square; Figure 3c). Note that we verified that phosphate buffer concentration (50-200 mM) had no effect on the rate of hydrazone formation whether arginine (400 mM) is present or not (see Figure S X). Thus, contrary to phosphate buffer, bicarbonate/CO₂ buffer plays a major role in the reaction and its combination with arginine enables to achieve rates as fast as $\sim 20 \text{ M}^{-1} \text{ min}^{-1}$.

In conclusion, arginine hydrochloride catalyzes hydrazone and oxime bond formation in water at neutral pH. The reaction can be performed in phosphate buffer but proceeds at a much faster rate in bicarbonate/CO₂ buffer. Arginine hydrochloride prevents the aggregation and precipitation of complex protein targets, is inexpensive, and shows low levels of toxicity. In that respect, arginine hydrochloride with its dual properties of nucleophilic catalyst and protein aggregation inhibitor is a useful addition to the hydrazone/oxime ligation toolbox.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, data fitting and characterization for all peptides (PDF)

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Author Contributions

NO, VA and OM wrote the manuscript. / NO, BS and RD performed the experiments. VA performed the data fitting. JV and OM supervised the work. OM conceived the study. / All authors have given approval to the final version of the manuscript.

Notes

§ *N,N*-dimethylaminoethylamine is classified as a corrosive and toxic substance with an LD50 in rat (1135 mg/kg) 10 fold lower than for arginine hydrochloride (12000 mg/kg). Source United States Environment Protection Agency

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