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Protein Chemistry

A Highly Efficient Strategy for Modification of Proteins at the C Terminus**

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Site-specific protein modification can facilitate the characterization of proteins with respect to their structure, folding, and interaction with other proteins both in biochemical and in cellular investigations. Although many chemical reactions are applicable in principle, methods for the site-specific modification of proteins remain in high demand, and there is a requirement for readily available ligation reagents and mild reaction conditions.[1] Oxime-based reactions have found wide application in the conjugation of biomolecules on account of the absence of oxyamino groups in proteins and their orthogonal reactivity with ketones to give stable oximes.[2–5] The oxyamine–ketone bioorthogonal reaction has been exploited in protein modification mainly by means of incorporating ketone groups into proteins by various chemical,[6] enzymatic,[7] and molecular biological[8] methods. To expand the application of this efficient methodology to protein ligation, we planned the development of simple and general methods to incorporate oxyamino groups into proteins. In an earlier approach to the use of auxiliary groups in native chemical ligation, it was shown that the nitrogen atom of oxyamines can react with a thioester group intramolecularly.[9] On the other hand, aminolysis of peptide and protein thioesters has been successfully reported in some studies.[10] We reasoned that in theory, highly nucleophilic oxyamines could also react with a thioester moiety in a protein intermolecularly. If this reaction would be performed with a linker carrying two oxyamino groups, then one oxyamine could form a hydroxamic acid bond to the protein and the second would still be available for a subsequent ligation reaction with the selectively functionalized protein. Herein, we describe how a bis(oxyamine) molecule was first incorporated at protein C termini to produce oxyamino-modified proteins, and how the subsequent efficient and specific reaction of the second oxyamino group with ketones was achieved to modify proteins site-specifically under mild conditions.

The unique position and chemistry of protein C termini has stimulated efforts to target this location for site-selective protein modification. In one approach, a protein tag is appended to a target protein, and an enzymatic reaction is used to covalently introduce a C-terminal modification onto a protein.[11–13] However, the protein tag is still retained in the labeled protein, which may interfere with protein function. An intein-based protein-cleavage reaction has generated very useful approaches to the C-terminal modification of proteins,[14] mainly based on thioester-mediated ligation chemistry.[15] A limitation of this method is that it generally leads to introduction of a cysteine residue into the target protein, regardless of whether this corresponds to the native structure or not. In addition, the thioester reaction frequently proceeds at relatively slow ligation rates and requires a relatively high protein concentration. Recently, it was reported that the C-terminal carboxylate can also be transformed into a thioacid, followed by C-terminal modification of the protein by means of thioacid/azide amidation in the presence of 6 M guanidine hydrochloride (Gdn-HCl) containing 3 mM 2,6-lutidine.[16] The modified conditions are too harsh to maintain the proper folding of a protein and the thioacid group is prone to hydrolysis. In contrast to these reports, we could introduce an oxyamino group into the C terminus of protein in phosphate buffer (pH 7.5), and the modified protein can subsequently undergo fast and chemoselective oxime ligation on ice.

The synthesis of 1,2-bis(oxyamino)ethane (**1**) started from the commercially available and inexpensive reagents *N*-hydroxyphthalimide and 1,2-dibromoethane (Figure 1).[17] Bis(oxyamine) **1** was obtained in a convenient manner without chromatography (> 20% yield over two steps; for details see the Supporting Information). The facile and economic synthesis of **1** is important for the wide use of the method reported herein. Rab1bΔ3-thioester (Ras-related GTPase) generated through intein-mediated partial protein splicing was chosen as the model protein. In order to introduce an oxyamino group into the protein, the reaction of Rab1bΔ3-thioester and **1** was performed on ice for 4 h (quantitative conversion). As shown in Figure 1, the bis-

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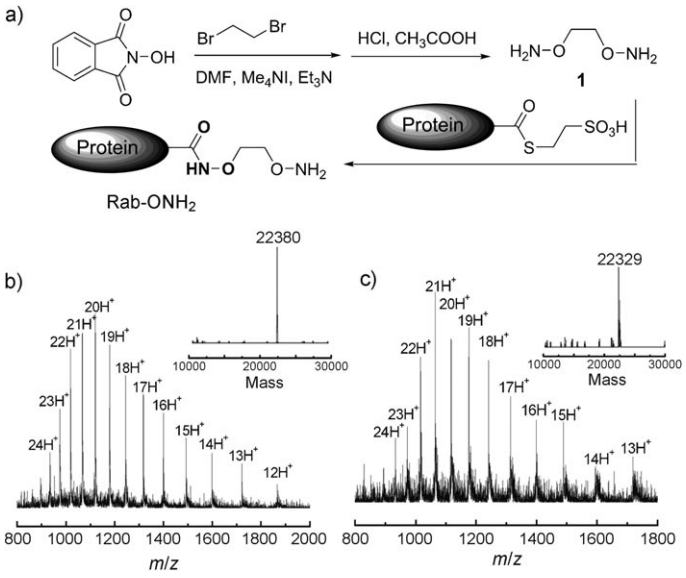

Figure 1. a) Synthesis of bis(oxyamine) **1** and the generation of Rab-ONH_2. b,c) ESI mass spectra of Rab1bΔ3 and Rab1bΔ3-ONH_2, respectively.

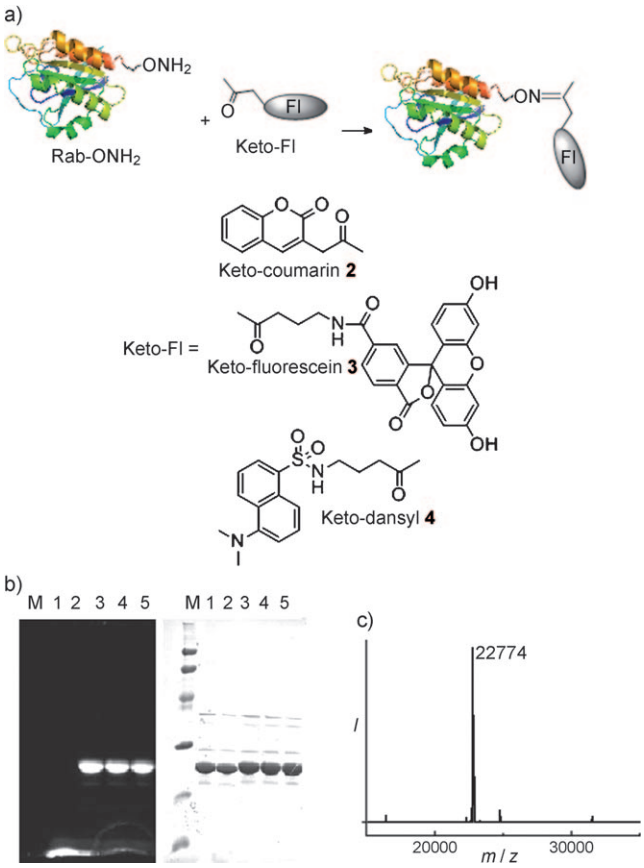

Figure 2. a) Fluorescence labeling at the C terminus by oxyamine–ketone ligation. b) Fluorescence (left) and Coomassie staining (right) images of a gel loaded with Rab1bΔ3-ONH_2 labeled with keto-fluorescein **3** (M: marker of 97, 66, 45, 30, 20.1, 14.4 kDa; lane 1: Rab1bΔ3-ONH_2; lane 2: Rab1bΔ3-thioester and keto-fluorescein for 30 min incubation on ice; lanes 3–5: reaction between Rab1bΔ3-ONH_2 and keto-fluorescein for 30 min, 1 h, 2 h on ice, respectively). c) ESI mass spectrum of the Rab1bΔ3–fluorescein ligation product.

(oxyamine) reacted directly with the thioester group to form a stable *O*-alkyl hydroxamic acid bond. ESI-MS analysis revealed that the main protein peak had a mass of 22329 Da (expected mass 22330 Da based on Rab1bΔ3), implying that most of the Rab1bΔ3 protein was modified with the oxyamino moiety. It should be noted that there is no sign of bis(oxyamino)-bridged proteins (see SDS-PAGE in Figure 2). There was also no detectable protein hydrolysis during this reaction. Unreacted **1** was removed from the Rab1bΔ3-ONH_2 by simple dialysis against sodium phosphate buffer (pH 7.5).

The reactivity of the oxyamino group on the protein was first tested by reaction with acetone. After simple incubation of Rab1bΔ3-ONH_2 (1 mg mL^{-1}) and acetone (10 mM) at pH 5.5 on ice, the reaction was finished within 20 min as shown by ESI-MS (Figure S3 in the Supporting Information), indicating that the reaction was rapid and generated a protein carrying a single acetone modification. In order to achieve fluorescent labeling of proteins, we first chose coumarin, since this fluorophore is highly soluble in water, has an excellent quantum yield, and is easy to synthesize (Figure 2).[18] On incubation of keto-coumarin **2** and Rab1bΔ3-ONH_2 on ice for 4 h, full conversion of the oxyamine to an oxime was achieved, as shown by ESI-MS. Excitation/emission scans of a solution of Rab1bΔ3–coumarin revealed that, as expected from the reporter group used, the fluorescence spectrum has an excitation maximum at 332 nm (data not shown) and an emission maximum at 412 nm (Figure S4 in the Supporting Information). In order to examine the efficiency of the fluorescent labeling by SDS-PAGE, keto-fluorescein **3** was synthesized and incubated with Rab1bΔ3-ONH_2 on ice for various reaction times. The fluorescence gel image (Figure 2b) showed that the reaction was rapid and complete within 1 h on ice. Rab1bΔ3-thioester did not show any labeling with keto-fluorescein **3**, further confirming that the reaction was bioorthogonal. The labeling reaction appeared to be highly efficient, since none of the original Rab1bΔ3-ONH_2 was detected by ESI-MS (Figure 2c).

To further validate this ligation method as a general method for C-terminal modification of proteins, it was also applied to enhanced yellow fluorescent protein (EYFP). As for Rab1b, the EYFP thioester was generated from an intein fusion protein, which was further modified with the oxyamino group by direct incubation of protein and bis(oxyamine) **1** in buffer. The obtained EYFP-ONH_2 was then incubated with keto-fluorescein **3** for different times on ice (Figure S6 in the Supporting Information). The fluorescence intensity of the band corresponding to the product increased with time, and the reaction was essentially complete after 30 min. Formation of the product with the expected mass was shown by ESI-MS.

In order to determine whether proteins still retain their activity after fluorescent labeling, we chose another Rab GTPase, Rab7, which binds to Rab escort protein (REP-1) with nanomolar affinity.[19] Fluorescence labeling of Rab7Δ2 was performed as for Rab1bΔ3 with keto-coumarin **2**. In the ESI mass spectrum the protein peaks have masses corresponding to Rab7Δ2-ONH_2 and Rab7-coumarin, 23273 and

23458 Da, respectively, consistent with the expected values of 23275 and 23459 Da (Table S1 in the Supporting Information). Rab7Δ2-coumarin and REP-1 were incubated on ice for 1 h, separated by gel filtration, and analyzed by SDS-PAGE. As shown in Figure S8 (Supporting Information), it is clear that Rab7-coumarin and REP-1 form a complex, implying that the modification of coumarin at the C terminus did not significantly perturb its interaction with REP-1.[20]

To assess whether this method can be used for studying protein–protein interactions, we attached an environmentally sensitive fluorescence probe, keto-dansyl **4**, to the protein C terminus by oxime ligation. When the semisynthetic Rab7Δ7-dansyl was titrated with increasing concentrations of REP-1, a dose-dependent and saturatable increase of the fluorescence emission signal at 500 nm was observed upon excitation at 340 nm (Figure 3). Titration data could be fitted

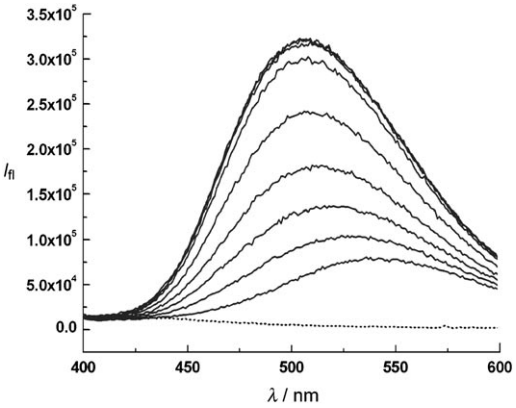

Figure 3. Emission spectra (excitation at 340 nm) of Rab7Δ7-dansyl (140 nM) upon addition of various concentrations of REP-1. Dotted line: blank, background. The gray lines represent the spectra obtained with various concentrations of REP-1 (0–861 nM, see the Supporting Information for details). The fluorescent signal increases with increasing concentration of REP-1.

using a quadratic equation describing the binding curve and were consistent with 1:1 stoichiometry (Figure S9 in the Supporting Information). The K_d value obtained (2.6 nM) is close to that determined previously using mant-GDP-bound Rab7 (1.0 nM).[21] Our results demonstrate that the oxyamine–ketone ligation offers an advantageous and novel approach for the site-selective functionalization and labeling of proteins for studies of protein–protein interactions.

In a further application of the method, we applied it to the generation of a doubly labeled Rab. Dual-color protein labeling is a valuable tool for fluorescence resonance energy transfer (FRET) studies of dynamic biomolecular dynamics and associations.[22] We expressed the protein mcherry-Ypt7Δ3-intein, since mcherry is a FRET acceptor for fluorescein. After performing the labeling reaction of mcherry-Ypt7Δ3-ONH_2 with keto-fluorescein **3** on ice for 2 h, we removed excess keto-fluorescein using NAP-5 columns (GE Healthcare). Fluorescence imaging of the gel shows dual-color labeling of the protein (Figure S10 in the Supporting Information). Figure 4 shows that FRET occurred

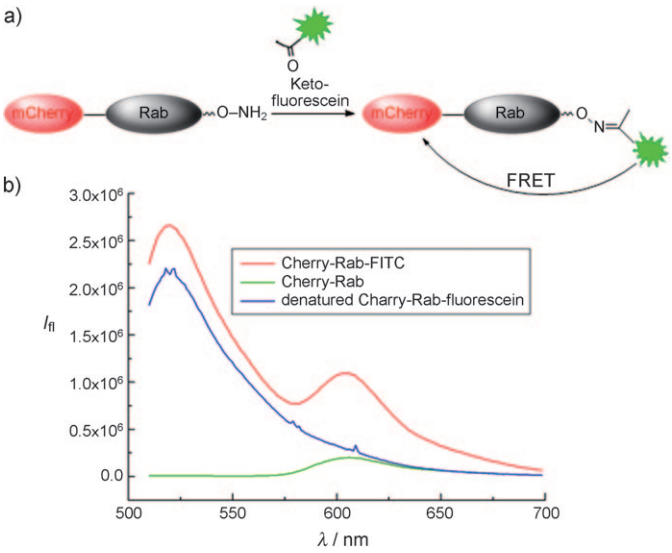

Figure 4. a) Schematic representation of the generation of a dual-color protein showing FRET between fluorescein and mcherry. b) Emission spectra of mcherry-Ypt7Δ3-fluorescein showing a FRET effect, while the denatured protein just shows the emission band of fluorescein.

within the mcherry-Ypt7Δ3-fluorescein molecule, since a strong fluorescent signal at 610 nm could be observed upon excitation at 490 nm (excitation wavelength for fluorescein). Compared with mcherry-Ypt7Δ3-ONH_2 at the same concentration and excited at the same wavelength, this represents a roughly sixfold increase in signal intensity due to FRET. In contrast, denatured mcherry-Ypt7Δ3-fluorescein displayed only a strong signal at the emission wavelength 510 nm, suggesting that the FRET disappeared because mcherry unfolded. FRET could provide a sensitive signal to test the folding state of the construct under the ligation conditions (i.e. at pH 5.5). Thus, storing mcherry-Ypt7Δ3-fluorescein in the ligation buffer for one week on ice did not lead to significant change in the fluorescence spectra (data not shown), further suggesting that the ligation conditions are mild enough for general protein modification, although this remains to be tested further in individual cases.

Oxime ligations normally proceed at modest reaction rates in acidic solution.[3b] The rate of oxime ligation in this study seems to be significantly faster than those reported when a keto group was incorporated into proteins.[9,23] For the five proteins in this study, all ligation reactions at pH 5.5 were complete within 4 hours on simple incubation of protein-ONH_2 and the ketone fluorophore on ice. Further experiments showed that the oxime reaction could also be achieved quantitatively at pH 6.5 (Figure S11 in the Supporting Information). Surprisingly, the ligation could be accomplished efficiently at pH 7.0 within 2 days by incubating 1 mg mL^{-1} Rab1Δ3-ONH_2 and 1 mM keto-fluorescein **3** on ice (Figure 5 and Figure S12 in the Supporting Information). Aniline can catalyze the oxime ligation in neutral buffer,[5a] and in our case the ligation rate can be further improved (by about fivefold) in the presence of 100 mM aniline at pH 7.0 (Figure 5).

We found that the oxime bond was stable enough at neutral pH to allow typical applications in biochemical

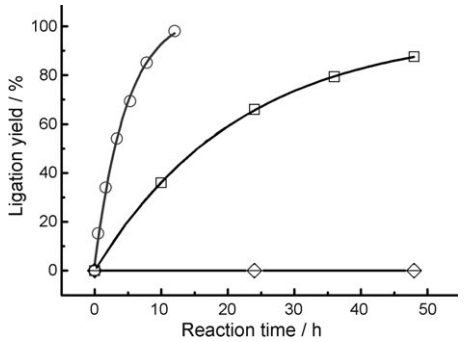

Figure 5. Reaction kinetics of 1 mg mL^{-1} Rab1bΔ3-ONH$_2$ and 1 mM keto-fluorescein **3** in the absence (□) or presence (○) of 100 mM aniline through incubation on ice in sodium phosphate buffer (pH 7.0) and the approximations of of the first-order reactions (solid lines). Rab1bΔ3CONHNH$_2$ did not react with keto-fluorescein under similar conditions (◇). The reaction half-lives are estimated to be 15.1 h and 2.8 h, respectively.

experiments (see the Supporting Information). Thus, the fast oxime ligation under mild conditions to form a stable oxime bond should provide a potentially general method for C-terminal modification of proteins.

It has been reported that the rate of the reactions between ketones and hydrazines or oxyamines is similar,[24] so we examined the reaction of Rab1bΔ3-CONHNH$_2$ (Figure 5 and Figure S12 in the Supporting Information) with keto-fluorescein **3** by hydrazone ligation. However, there was no detectable ligation product after incubation of 1 mg mL^{-1} Rab1bΔ3-CONHNH$_2$ and 1 mM keto-fluorescein for 24 h on ice at pH 5.5, implying that the hydrazone ligation is much slower than the oxime ligation we describe here.

It is plausible that the particular functional moiety introduced at the protein C terminus might contribute to the fast oxime ligation kinetics. Kinetic studies based on a model molecule in which the same dioxyamine function was added to Fmoc-lysine (Figure S14 in the Supporting Information) were performed in pH 7.0 buffer (Figures S15 and S16 in the Supporting Information). The oxime reaction with the model molecule was about tenfold faster than that for *O*-benzylhydroxylamine, implying that our oxyamine is a particularly effective nucleophile for rapid reaction with ketones in protein modification.

In conclusion, we report a facile, chemoselective, and potentially general method for the C-terminal modification of proteins based on a fast oxyamine–ketone ligation. As a useful alternative to existing approaches to protein labeling, the rapid reaction of the oxyamino-modified proteins with ketones under mild condition is intriguing and potentially advantageous for many biological applications. Recent advances in protein thioester synthesis[25] could provide further access to oxyamino-modified proteins, thus helping to extend the application of our method to areas such as protein immobilization on keto-modified surfaces.

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