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Complex Acylation of Angiotensin II by N-Hydroxysulfosuccinimide linked Biotin Reagents

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Abstract

N-Hydroxysulfosuccinimide-linked biotins (sulfoNHS-biotins) are water soluble biotin tags commonly used to conjugate a biotin moiety to proteins by rapid N-acylation of primary amines. Unexpected O-acylation by sulfoNHS-biotin on tyrosine of Angiotensin II (Ag-II) and an acylation on third site unable to characterize were identified by LC-MS in addition to the expected N-acylation on the N-terminal of Ag-II. The N-acylation only undergoes incomplete hydrolysis in 0.1% formic acid not at pH 7.2 and 8.0, while two unexpected acylation hydrolyze at both conditions, but their hydrolysis in 0.1% formic acid was much more rapid. Dithiothreitol treatment selectively catalyzed hydrolysis of both of the unexpected acylation but not the N-acylation of Ag-II. The maximum yield of O-acylation of the Ag-II tyrosine was 99% at pH 7.2 and 95% at pH 8.0 as compared N-acylation of lysine when reacted with excess sulfoNHS-biotin with these yields of 94% at pH 7.2 and 96% at pH 8.0. Acylation of the third uncharacterized site of Ag-II showed maximum yield of approximately 17% at pH 7.2, but higher yield ($\geq$ 47%) at pH 8.0 within 30 min. The unexpected O-acylation of the Ag-II tyrosine occurred within 1 min at either pH 7.2 or pH 8.0, as rapidly as the N-acylation, while the other unexpected acylation required more time to complete at pH 8.0.

Keywords: Angiotension II; SulfoNHS-biotin; Acylation; Hydrolysis; LC-MS

Abbreviations: SulfoNHS-biotins: N-Hydroxysulfosuccinimide-linked biotins; Ag-II: Angiotensin II; NHS: N-hydroxysuccinimide; Myc: Peptide EQKLISEEDL

Introduction

Labeling proteins with a molecular tag has been a standard way to capture or detect proteins in the study of cellular events; such as, intracellular transport, synthesis, processing, secretion and degradation. N-hydroxysulfosuccinimide linked biotins (sulfoNHS-biotins) are one type of popular tagging reagent for covalently attaching a biotin molecule to a protein or other molecule [1]. They contain an amine reactive group in one end, a biotin on the other end, and a spacer arm in between to reduce steric hindrance of the tagged protein that may interfere with subsequent binding of the biotin by streptavidin. The biotin moiety would be good for affinity isolation or detection of tagged proteins using biotin-avidin affinity technology. N-hydroxysuccinimide (NHS) ester is amine-reactive acylating agent that couples principally with the primary amines. The charged sulfonate ($-SO_3^-$) group on the NHS ring has no effect on the reaction chemistry, but makes the reagents water soluble and prevents sulfoNHS esters from permeating cell membranes. Therefore, application of sulfoNHS-linked biotins is often used to label proteins in aqueous conditions, particularly cell-surface proteins, for various purposes in proteomic research [2]. The sulfoNHS moiety rapidly reacts with ε-NH_2 of lysine side chains and the α-NH_2 of the N-terminal amino acid of a peptide or protein, under slightly alkaline conditions (pH 7.0-8.5) to yield stable amide bonds [3].

Angiotensin II (Ag-II) is a peptide hormone converted from Angiotensin I through the removal of two C-terminal residues by angiotensin-converting enzyme. It plays key roles in management of blood pressure and body fluid retention via the renin-angiotensin system [4]. The sequence of Ag-II - DRVYIHPF- contains only one primary amine, which is the α-amine of the N-terminus. When Ag-II is labeled by sulfoNHS-biotin, a single biotinylation product was expected, but, rather unexpectedly, a rapid dibiotinylation and a tribiotinylation were observed. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to characterize the di- and tribiotinylated Ag-II, which determined that the dibiotinylation occurs on the tyrosine residue through O-acylation in addition to the N-terminus though O-acylation conjugate is generally not favored due to competition from hydrolysis in an aqueous environment [3]. However, site for the third biotinylation was unable to identify through tandem MS. Stability of three acylation of Ag-II was examined under conditions commonly used for proteomic sample preparation. Since lysine is the expected sulfoNHS-biotin conjugation site of proteins, a peptide containing a lysine residue (EQKLISEEDL, known as the Myc tag) was, also, biotinylated; and characterized to compare the reactivity of tyrosine and unknown site of Ag-II with lysine residues.

Two commercially available sulfoNHS linked biotin reagents were used in this study, as shown in Figure 1. SulfoNHS-SS-biotin contains a thiol-cleavable disulfide bond and sulfoNHS-LCLC-biotin does not.

Materials and Methods

Chemicals

SulfoNHS-LCLC-Biotin and SulfoNHS-SS-Biotin were purchased from Thermo Fisher Scientific. Ag-II (DRVYIHPF), Myc peptide (EQKLISEEDL), Dithiothreitol (DTT), iodoacetamide (IAM) and formic acid (FA) were purchased from Sigma-Aldrich. HPLC-grade acetonitrile and water were from VWR. All other chemicals were obtained from commercial sources at HPLC- or reagent-grade.

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Figure 1: Chemical structures of sulfoNHS-SS-biotin and sulfoNHS-LCLC-biotin.
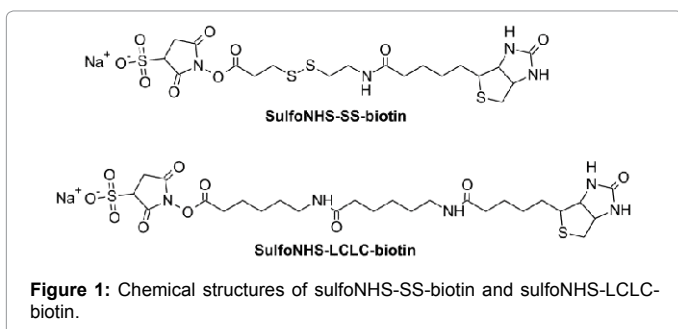
Biotinylation procedure

Peptides were dissolved in 0.1 M PBS buffer, pH 7.2 or pH 8.0 to make 1 µg/µL solutions, then 10 mM sulfoNHS-LCLC-Biotin (6.7 µg/µL) or 10 mM sulfoNHS-SS-Biotin (6.1 µg/µL) in deionized water was added at the molar ratio of 40:1 (biotin/ primary amines of the peptide) unless specified otherwise. Each mixture was incubated for a time as specified in Results/Discussion. The same volume of 50 mM Tris buffer, pH 8.0 was added to the reaction mixture to provide excess of primary amine to react with the remaining sulfoNHS-linked biotin. The quenched mixture was ×50 diluted with water or treated with DTT/IAM before dilution, and then injected into LC-MS.

DTT treatment

A stock solution of DTT (500 mM in water) was added to the Tris-quenched biotinylated peptides to a final concentration of 10 mM and incubated at 55°C for 20 min.

IAM alkylation

A stock solution of IAM (100 mM in water) was added to DTT-reduced peptides to a final concentration of 20 mM and incubated at room temperature in the dark for 20 min.

LC-MS analysis

HPLC was carried out on a UFLC system (Shimadzu) with binary pumps equipped with a Symmetry C18 column, (5 cm × 2.1 mm, particle size 3.5 µm, Waters). Mobile phase A was water and B was acetonitrile, both containing 0.1% FA. The flow rate was maintained at 0.4 mL/min. Injection volumes varied from 1 to 50 µL, to load approximately 0.1 µg of total peptides. Samples were eluted with gradients specified in each chromatogram. An Ion Trap mass analyzer equipped with an ESI probe (LCQ Fleet, Thermo Scientific) was used to collect mass spectra in positive mode. The ESI-MS was tuned using direct infusion of Angiotensin II prior to LC-MS analysis. LC-MS chromatogram and MS spectra were analyzed using Xcalibur SP2.0.7. Theoretical b and y fragments were calculated using the online MS-product tool.

Results and Discussion

### Double biotinylation of Ag-II by SulfoNHS-SS-biotin

SulfoNHS-SS-biotin is probably the most frequently used sulfoNHS-biotin for enabling biotin-avidin technology, because the thiol-cleavable disulfide bond in the spacer arm allows easy removal of the biotin label by treatment with 1-10 mM dithiothreitol (DTT). This means tagged and captured proteins can be easily released without breaking the extremely strong biotin-streptavidin binding [3]. When Ag-II was reacted with equimolar sulfoNHS-SS-biotin at pH 7.2 for 30 min, the LC-MS chromatograms showed that Ag-II was only partially biotinylated (Figure 2, 1:1 ratio) with a significant amount of Ag-II (peak A, m/z 523.8, z = +2 and m/z 349.5, z = +3) left unmodified. The major products were two isomers of monobiotinylated Ag-II (peaks

B and C, both having a mass of m/z 718.3, z = +2 and m/z 479.2, z = +3) with dibiotinylated Ag-II (peak D, m/z 912.9, z = +2 and m/z 608.9, z = +3) as a minor product. When the molar ratio of sulfoNHS-SS-biotin/Ag-II was increased to 5:1 and above, the unmodified Ag-II dramatically declined. Ag-II (DRVYIHPF) contains only one primary amine, which is the N-terminal α-amine. SulfoNHS-SS-biotin was expected to attach to this site and form a single N-acylation product. The location for the unexpected second biotinylation of Ag-II was, therefore, not easy to predict. Recognized as amine-specific reagents, sulfoNHS esters are occasionally reported to attach to the guanidino group of arginine, as well as, O-acylating hydroxyl-containing residues; such as serine, threonine, and tyrosine, especially when they were located two positions away on either side of a histidine residue [5-7]. The peptide sequence of Ag-II fits the YXH motif, but the corresponding O-acylation is usually considered unstable in aqueous environments due to immediate hydrolysis.

When the Ag-II and sulfoNHS-SS-biotin reaction mixture (molar ratio of sulfoNHS-SS-biotin/Ag-II = 10:1) was incubated with 10mM DTT for 20 min at 55°C, the major product was expected to be DRVYIHPF(-COCH2CH2SH)$_2$ generated from peak D in Figure 2, but only D(-COCH$_2$CH$_2$SH)RVYIHPF was produced, as determined by its MS^2 spectrum (Figure S1). That means one acylation of Ag-II was lost upon the DTT treatment. The alkylation of the sulfhydryl group in D (-COCH$_2$CH$_2$SH) RVYIHPF by IAM gave a yield of approximately 50%, even when excess amounts of IAM and extended incubation time were used (Figure S2). The incomplete alkylation of the free thiols may result in protein-protein crosslinking and interfere with subsequent data analysis in typical proteomic workflows, especially for complex samples and crosslinking studies. Therefore, a non-cleavable sulfoNHS-LCLC-biotin was switched to instead of sulfoNHS-SS-biotin for further study.

MS/MS characterization of biotinylation of Ag-II

When Ag-II reacted with sulfoNHS-LCLC-biotin at pH 7.2 for 30 min at an equimolar ratio; results similar to those shown in Figure 2 were obtained. Some Ag-II was left un-biotinylated due to the fact that the biotinylation reagent was limiting in the reaction (Figure 3A). Two isoforms of monobiotinylated Ag-II with a dibiotinylated Ag-II were also produced. Theoretical fragments (b and y ions) through collision-induced dissociation (CID) of the peptide with biotinylation at each residue can be predicted [8]. By matching tandem MS^2 spectra to the predicted fragments (Table S1), biotinylation of the first eluted monobiotinylation isomer can be unambiguously assigned to the tyrosine residue, while the later eluted isomer was biotinylated on
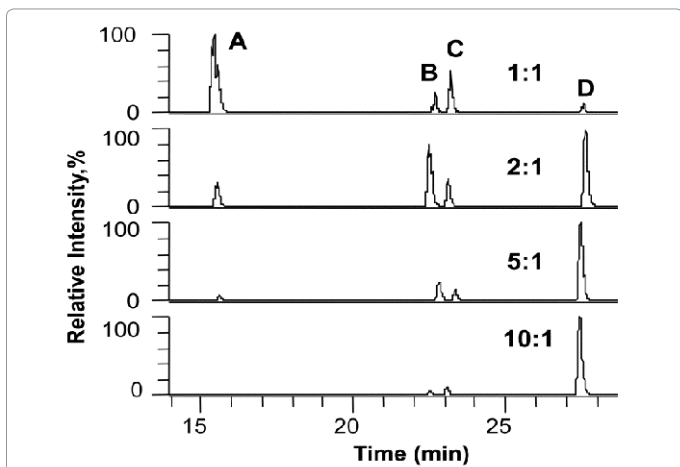

Scheme 1: Acylation of Ag-II by SulfoNHS-LCLC-biotin and the hydrolysis of the products.
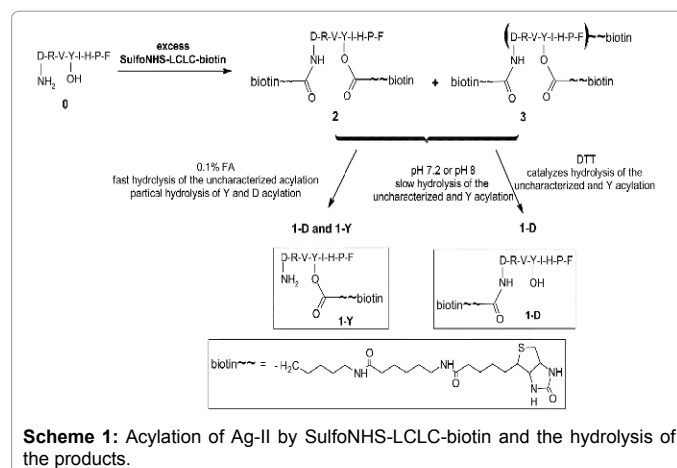
Figure 2: LC-extracted ion chromatograms of Ag-II reacted with sulfoNHS-SS-biotin at pH 7.2 for 30 min. A linear gradient elution (2-25% B over 30 min) was performed. From top to bottom the molar ratio of sulfoNHS-SS-biotin/Ag-II was 1:1, 2:1, 5:1, 10:1. Peak A – unlabeled Ag-II; Peaks B and C – two isomers of singly biotinylated Ag-II; Peak D – doubly biotinylated Ag-II.
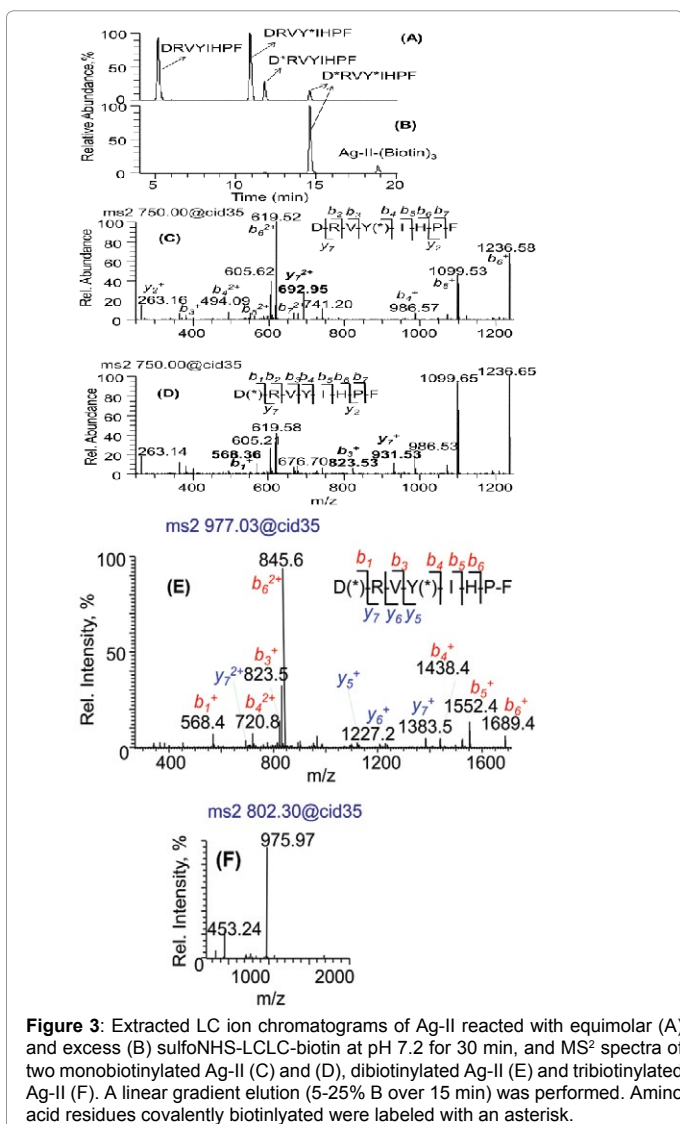

Figure 3: Extracted LC ion chromatograms of Ag-II reacted with equimolar (A) and excess (B) sulfoNHS-LCLC-biotin at pH 7.2 for 30 min, and MS² spectra of two monobiotinylated Ag-II (C) and (D), dibiotinylated Ag-II (E) and tribiotinylated Ag-II (F). A linear gradient elution (5-25% B over 15 min) was performed. Amino acid residues covalently biotinlyated were labeled with an asterisk.

the N-terminal aspartate (Figures 3C and 3D). The dibiotinylation product was D (LCLCbiotin) RV-Y (LCLCbiotin) IHPF, in which both the N-terminal and tyrosine residues were acylated according to CID fragments of its MS² (Figure 3E). The larger peak area from monobiotinylation of tyrosine confirmed a higher yield than for that on the N-terminus. This indicates the hydroxyl on the tyrosine of free Ag-II is even more reactive than the α-amine of the aspartate N-terminus for acylation by sulfoNHS-LCLC-biotin.

When Ag-II reacts with excess sulfoNHS-LCLC-biotin (mole ratio of Ag-II: sulfoNHS-LCLC-biotin = 40:1), unmodified and both monobiotinylated Ag-II disappeared while dibiotinylated Ag-II became the dominant product. In addition a tribiotinylation of Ag-II was surprisingly observed as shown in Figure 3B that were not observed in case of sulfoNHS-SS-biotin. However position of the third acylation was not able to identify because CID exclusively fragmented at the third acylation giving two daughter ions corresponding to the dibiotinylated Ag-II (m/z 976) and acylation moiety from sulfoNHS-LCLC-biotion (m/z453) (Figure 3F). Abello et al reported a triply acylated Ag-II by reacting with excess acetic acid N-hydroxysuccinimide ester and the third acylation was on the arginine residue identified by tandem MS [9].

In order to understand if tyrosine biotinylation also occurs on proteins that contain a large number of tyrosine, the biotinylation of a readily available protein - BSA was examined. When BSA reacted with excess sulfoNHS-LCLC-biotin (molar ratio of biotin/ primary amines of BSA = 40:1) at pH 8.0 for 30 min, biotinylation on 17 lysines out of 58 lysine residues of BSA was identified, but no tyrosine biotinylation was detected even though BSA sequence has one HXY motif-containing tryptic peptide (HPYFYAPELLYYANK) (Data not shown). Therefore O-acylation on tyrosine by sulfoNHS-LCLC-biotin might be sequence dependent and not a general phenomenon for all proteins.

Stability of biotinylated Ag-II

LC-MS/MS data confirmed that when Ag-II reacts with excess sulfoNHS-LCLC-biotin at pH 7.2, it is mainly double biotinylated through *N*-acylation of the N-terminal primary amine to form an amide bond; and simultaneous O-acylation of the tyrosine hydroxyl group to form an ester bond, plus a minor tribiotinylated Ag-II with a third acylation at an unidentified position and trace amount of monobiotinylated Ag-II. Both esters and amides can undergo hydrolysis in aqueous solutions. Therefore, stability of all observed biotinylated Ag-II was examined under multiple conditions including storage in 0.1% formic acid (FA), 1 × PBS pH 7.2 or 1 × PBS pH 8.0 at 4°C for 1 day and 10 days, and incubation at 55°C for 20 min at pH 8.0 with or without DTT. These conditions were investigated because they are commonly used in proteomics sample preparation. Figure 4 shows the relative percentage of each biotinylated Ag-II under various conditions.

Results in Figure 4 indicate the third uncharacterized acylation of Ag-II was not stable at all three pH conditions. It was completely lost immediately in 0.1% FA solution and 10 days at pH 7.2 and pH 8.0 phosphate buffers. 0.1% FA catalyzes partial hydrolysis of both O-acylation of tyrosine and N- acylation of the N-terminal of Ag-II, and the resulting equilibrium produced no further increase when time was extended from 1 h to 24 h (data not shown) and then to 10 days. Both pH 7.2 and pH 8.0 only catalyzed the hydrolysis of the O- acylation of tyrosine, and more hydrolysis was observed in pH 8.0 than in pH 7.2 at 10 days. But, the process was slower in both pH 7.2 and pH 8.0 when compared to the hydrolysis in 0.1% FA, and it was observed that the alkaline hydrolysis continued as time increased until all O-acylation of
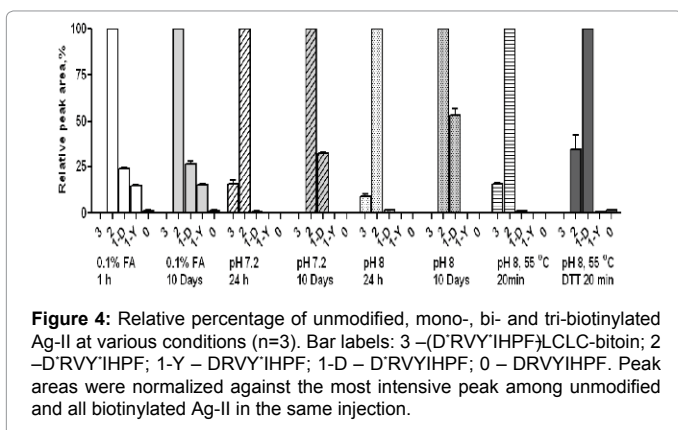
Figure 4: Relative percentage of unmodified, mono-, bi- and tri-biotinylated Ag-II at various conditions (n=3). Bar labels: 3 –(D˚RVY˚IHPF)LCLC-bitoin; 2 –D˚RVY˚IHPF; 1-Y – DRVY˚IHPF; 1-D – D˚RVYIHPF; 0 – DRVYIHPF. Peak areas were normalized against the most intensive peak among unmodified and all biotinylated Ag-II in the same injection.
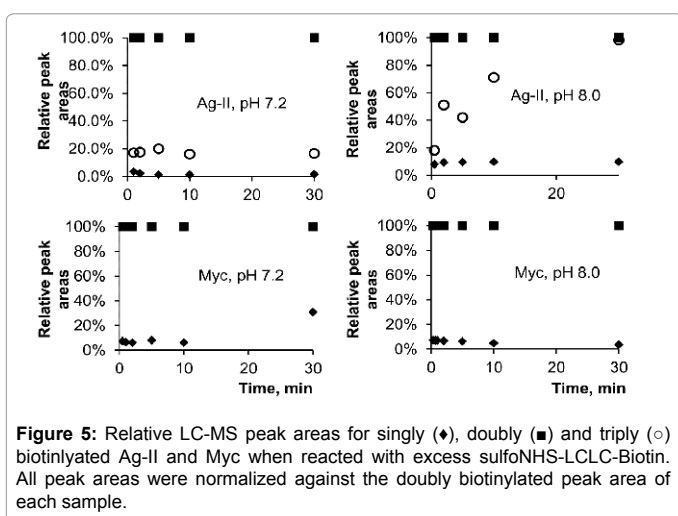

Figure 5: Relative LC-MS peak areas for singly (♦), doubly (■) and triply (○) biotinlyated Ag-II and Myc when reacted with excess sulfoNHS-LCLC-Biotin. All peak areas were normalized against the doubly biotinylated peak area of each sample.

tyrosine was eventually consumed, which can take more than 1 month at 4°C (data not shown). These results agree with the general mechanism of ester hydrolysis, which can be promoted by both acid and base [10]. The reaction is generally reversible when catalyzed by acid but irreversible when it occurs under alkaline conditions. Interestingly, DTT treatment at 55°C for 20 min completely removed the uncharacterized third acylation of Ag-II by SulfoNHS-LCLC-biotin and accelerated hydrolysis of the O-acylation of tyrosine. On the contrary the N-acylation was not affected, since the second monobiotinylated Ag-II (1-D), as shown in Figure 3A, remained and became predominately produced while no DRVY˚IHPF (1-Y) was detectable upon DTT treatment. It was confirmed that the elevated temperature up to 55°C alone that is typically used during a DTT treatment in proteomic sample treatment was not the reason for the enhanced hydrolysis (pH 8, 55°C as shown in Figure 4), which is different from observations at temperature of boiling water [9]. Therefore it was concluded DTT catalyzed hydrolysis of the ester formed by acylation of Ag-II at the tyrosine residue and the uncharacterized third acylation with sulfoNHS-LCLC-biotin. The complex acylation of Ag-II by sulfoNHS-LCLC-biotin and stability of all detected biotinylated Ag-IIs were summarized in Scheme 1.

Comparison of acylation reactivity at different sites

SulfoNHS-biotin reagents are commonly expected to covalently tag proteins by reacting with lysines. It is interesting to know the relative reactivity of tyrosine, N-terminal and uncharacterized site of Ag-II compared to lysine with regard to biotinylation by this type

of reagents. For this purpose, sulfoNHS-LCLC-biotin was used to compare biotinylation of EQKLISEEDL (Myc), a peptide containing a lysine residue, with that of Ag-II. Myc contains two reactive amines: one on the lysine side chain and the other being the α-amine of the N-terminal glutamic acid. Therefore, dibiotinylation is expected for Myc. No O-biotinylation on the serine of Myc was detected, even though serine has hydroxyl group as tyrosine. Unlike Ag-II, Myc only showed one monobiotinylation product with the modification on the N-terminus when Myc was reacted with equimolar sulfoNHS-LCLC-biotin, in addition to a dibiotinylation product in which the N-terminus and lysine were both tagged (Figure S3). This indicates the lysine residue of Myc is less reactive than the N-terminus, but it cannot be determined if this is peptide sequence-dependent.

When both Ag-II and Myc were reacted with large excess of sulfoNHS-LCLC-biotin at pH 7.2 or pH 8.0 for up to 30 min, no detectable unmodified Ag-II or Myc was observed even at the shortest reaction time points while the doubly biotinylated products were predominant and the yield of the singly biotinylated products did not change much (Figures S4 and S5). Figure 5 showed the relative peak areas of the singly, doubly and triply (Ag-II only) biotinylated Ag-II and Myc during the reaction monitored by LC-MS analysis. The reaction was very rapid (<1 min) for acylation of all sites including lysine of Myc, the N-terminal of both peptides, tyrosine and the third uncharacterized site of Ag-II. A trace amount of monobiotinylation was detected for both Ag-II and Myc, in which the biotinylation was on the N-terminus with the tyrosine and lysine remaining unmodified. This was confirmed by retention time alignment with monobiotinylated Ag-II or Myc identified in equimolar reaction mixtures. Therefore, the attachment of the second biotin moiety was more favored towards N-terminals, than either lysine or tyrosine. However, Figure 3A indicates the tyrosine of free Ag-II is more reactive than the N-terminus, so it is likely that reactivity or reaction equilibrium of the modification site of Ag-II is affected by modifications that have already occurred in the vicinity. Overall, the observed maximum yield of O-acylation of the Ag-II tyrosine was approximately 99% of at pH 7.2 and 95% at pH 8.0, while N-acylation on the N-terminus was complete for both Ag-II and Myc. N-acylation on the lysine residue of Myc exhibited slightly better maximum yield at pH 8.0 (96%) than that at pH 7.2 (94%). Moreover, the yield of the N-acylation on lysine at pH 7.2 dropped significantly (<85%) when the reaction time was over 30 min, which indicates pH 8.0 is more favorable for using sulfoNHS-linked esters to tag lysines. Acylation of the third uncharacterized site of Ag-II showed maximum yield of approximately 17% at pH 7.2, but much higher yields at pH 8.0 which kept increasing to 47% at 30 min, and the tribiotinylated Ag-II could exceed the dibiotinylated Ag-II becoming the predominant product as the reaction was further extended. All yield percentage was normalized against the yield of dibiotinylated Ag-II or Myc. This finding demonstrates the importance of pH in peptide/protein conjugation using sulfoNHS-biotin esters and storage conditions of the biotinylated samples.

Conclusion

Two unexpected acylations on tyrosine and an uncharacterized site of Ag-II by sulfoNHS-biotin reagents were identified by LC-MS besides the expected N-acylation of primary amine on its N-terminal residue. When Ag-II reacted with excess sulfoNHS-LCLC-Biotin, the unexpected O-acylation biotinylation of tyrosine occurred within 1 min as rapidly as the N-acylation. The other unexpected acylation exhibited lower yields. We also found that two unexpected acylations can be selectively hydrolyzed slowly under mild basic storage conditions and

catalyzed by dithiothreitol treatment while the N-acylation remains. However both unexpected and expected acylations exhibit fast hydrolyze in 0.1% formic acid acidic solution.

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