Differentiation of isobaric cross-linked peptides prepared via maleimide chemistry by MALDI-MS and MS/MS.

Toshifumi Takao¹, Luis Gonzalez², Satomy Pousa², Hironobu Hojo¹, Shio Watanabe³, Daisuke Higo³, and Alina Rodriguez Mallon²

¹Osaka Daigaku Tanpakushitsu Kenkyujo
²Centro de Ingenieria Genetica y Biotecnologia
³Thermo Fisher Scientific Inc Mitato-ku Tokyo

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Abstract

RATIONALE: The thiosuccinimide linker is widely used in the synthesis of bioconjugates. However, it is susceptible to hydrolysis and is transformed into its hydrolyzed and/or the isobaric thiazine forms, the latter of which is a fairly common product in a conjugate that contains a cysteinyl peptide. MALDI-MS and MS/MS are useful for differentiating these isobaric species. METHODS: Four cross-linked peptides with thiosuccinimide linkers were synthesized. Analogs with the linker that were transformed into thiazine and/or the hydrolyzed thiosuccinimide linkers were then generated by incubating the samples at neutral or basic pH. All of the cross-linked peptides were purified by rp-HPLC and differentiated by MALDI-MS, -MS/MS and UVPD. RESULTS: A cysteinyl peptide-containing conjugate, the thiosuccinimide form, was largely transformed into the hydrolyzed or thiazine forms after incubation at neutral or basic pH. MALDI-MS allowed the three forms to be differentiated: the thiosuccinimide and its hydrolysis product gave two constituent peptides after reductive cleavage between the Cys and succinimide moieties; no fragment ions were produced from the thiazine form. In addition, MALDI-MS/MS of the thiosuccinimide form yielded two pairs of complementary fragment ions via 1,4-elimination: Cys-SH and maleimide, and dehydro-alanine and thiosuccinimide, which are different from those produced via reductive cleavage in MALDI-MS. The thiazine form gave fragment ions resulting from the cleavage of the newly formed amide bond in the linker that arose from thiazine formation. CONCLUSIONS: The thiosuccinimide (but not thiazine) form of the cross-linked peptide yielded individual constituent peptides in MALDI-MS; MALDI-MS/MS showing specific 1,4-elimination for the thiosuccinimide form and cleavage at the newly formed peptide bond via transcyclisation for the thiazine form.

Title: Differentiation of isobaric cross-linked peptides prepared via maleimide chemistry by MALDI-MS and MS/MS.

Short Title: Differentiation of thiazine and thiosuccinimide linkers by MS.

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Authors: Luis Javier González¹,*,#, Satomy Pousa¹,#, Hironobu Hojo², Shio Watanabe³, Daisuke Higo³, Alina Rodriguez Mallon⁴ and Toshifumi Takao⁵,#

Affiliations:

2Laboratory of Protein Organic Chemistry. Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan.


5Laboratory of Protein Profiling and Functional Proteomics, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan.

(*) Authors with equal contributions

(§) Corresponding authors:

Dr. Toshifumi Takao
Laboratory for Protein Profiling and Proteomics, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
Phone: +816-6879-4312
Email: tak@protein.osaka-u.ac.jp

Dr. Luis Javier González López
Email: luis.javier@cigb.edu.cu

Abstract:

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METHODS: Four cross-linked peptides with thiosuccinimide linkers were synthesized. Analogs with the linker that were transformed into thiazine and/or the hydrolyzed thiosuccinimide linkers were then generated by incubating the samples at neutral or basic pH. All of the cross-linked peptides were purified by rp-HPLC and differentiated by MALDI-MS, -MS/MS and UVPD.

RESULTS: A cysteinyl peptide-containing conjugate, the thiosuccinimide form, was largely transformed into the hydrolyzed or thiazine forms after incubation at neutral or basic pH. MALDI-MS allowed the three forms to be differentiated: the thiosuccinimide and its hydrolysis product gave two constituent peptides after reductive cleavage between the Cys and succinimide moieties; no fragment ions were produced from the thiazine form. In addition, MALDI-MS/MS of the thiosuccinimide form yielded two pairs of complementary fragment ions via 1,4-elimination: Cys-SH and maleimide, and dehydro-alanine and thiosuccinimide, which are different from those produced via reductive cleavage in MALDI-MS. The thiazine form gave fragment ions resulting from the cleavage of the newly formed amide bond in the linker that arose from thiazine formation.

CONCLUSIONS: The thiosuccinimide (but not thiazine) form of the cross-linked peptide yielded individual constituent peptides in MALDI-MS; MALDI-MS/MS showing specific 1,4-elimination for the thiosuccinimide form and cleavage at the newly formed peptide bond via transcyclisation for the thiazine form.

Keywords:
Introduction:

The thiol-maleimide reaction \(^1\) also known as the thiol-Michael addition \(^2\), is included among the chemical reactions classified as click-chemistry \(^2,3\) and is one of the most widely used methods for conjugation \(^4,5\) and the synthesis of antibody drug conjugates (ADC) and conjugate vaccines \(^4\).

As a result of this chemical reaction, the cytotoxic drug and the antigen are linked to the carrier protein through a thioether bond, generally between a cysteine residue, a five-membered succinimide ring and an \(N\)-acyl group that is linked to primary amino groups (structure I, Figure 1). The resultant five membered thiosuccinimide ring is considered to be unstable \(in\ \text{vivo}\) due to the fact that a retro-Michael addition can occur \(^6,7\) (Figure S1). In serum, the presence of molecules with free thiols (e.g. free Cys, reduced glutathione, albumin, etc.), through a thiol-exchange reaction, promotes the release of the antigen or the cytotoxic drug linked to the thiosuccinimide ring in the conjugate vaccine and ADC, respectively. This reaction, which is undesirable for the development of a biotechnology product, increases the cytotoxicity of the ADC \(^8\), and it could also lead to the partial or complete loss of the biological activity of the conjugate vaccine. To overcome this potential \(in\ \text{vivo}\) instability of the resultant five-membered thiosuccinimide ring, it can be stabilized through hydrolysis \(^9-13\) or via Michael-transcyclization reactions \(^14,15\).

Hydrolysis of the five-membered thiosuccinimide could proceed through two pathways (see pathways (a) and (b) in structure (I), Figure 1) that would yield two thiosuccinamic acid isomers (structures (II) and (III), Figure 1) \(^10\). These isomers are stable \(in\ \text{vivo}\) and are not subject to the retro Michael addition. This hydrolysis of the thiosuccinimide linker proceeds more rapidly at basic pH, and also takes place during the sample preparation and proteolysis of conjugates regardless of the position of the cysteine residue that is involved in the thioether bond \(^16-18\). The thiosuccinamic acids, hydrolysis products derived from a thiosuccinimide, can be readily identified by the MS-mode, based on the fact that the molecular masses of these species differ by 18 Da.

In the case of the peptide with a free cysteine at the \(N\)-terminus, the transcyclization predominantly takes place by a nucleophilic attack of the amino terminal group of the cysteine residue to the carbonyl group in the five-membered ring of the thiosuccinimide, which, through an internal rearrangement, results in the formation of a more stable six-membered thiazine ring and a new pseudopeptide bond as well (structure (IV), in Figure 1) \(^14\).

The conjugate peptides linked through thiazine or thiosuccinimide are isobaric, and thus, cannot be differentiated based on their molecular masses. However, the thiazine form can be identified by CID or HCD fragmentation \(^15\). This is because the thiazine form can undergo cleavage at new pseudo-peptide bond that was formed through the transcyclization process \(^14\) (see structure (IV), in Figure 1).

The findings reported in this study show that C-S linkage between the cysteine sulfur atom and the succinimide at the conjugation site is amenable to cleavage by MALDI-MS, MALDI-MS/MS or UVPD/HCD-MS/MS, which yields two component peptides, namely, a free-thiol-containing peptide and its counterpart linked to the original \(N\)-propionyl maleimide and \(N\)-propionyl succinimide groups. The thiazine form, however, is stable and no fragment ions derived from it are produced in MALDI-MS, but fragment ions for the newly formed peptide bond are observed in MALDI-MS/MS.

Materials and Methods:

Peptide Synthesis

Starting from the Fmoc-Lys(Boc)-OCH\(_2\)-Wang resin (0.175 g, 0.1 mmol, 0.57 mmol/g), the peptide was elongated by CEM Liberty Blue using a 0.1 mmol scale protocol to give an Fmoc-AAGGGAAAAK(Boc)-resin. The resulting resin was divided into two portions and one was used for the further synthesis to obtain the Fmoc-AAAGGGAAAAK-resin (P1 peptide). For the other, Fmoc-C(Trt)-OH (0.25 mmol act by HBTU
0.25 mmol, DIEA 0.5 mmol) was reacted for 30 min at 50 °C to give the Fmoc-C(Trt)AAGGGAAAAK(Boc)-resin (P2 peptide).

Starting from the Fmoc-Arg(Pdf)-OCH$_2$-PEG-resin (0.23 g, 0.05 mmol, 0.22 mmol/g), the peptide chain was elongated by CEM to obtain Ac-GAN(Trt)APK(Boc)E(OBut)PQ(Trt)R(Pbf)-OCH$_2$-PEG-resin (P4 peptide). Starting from the Fmoc-Lys(Boc)-OCH$_2$-Wang resin (0.0875 g, 0.05 mmol, 0.57 mmol/g), the peptide chain was elongated by CEM to obtain NH$_2$-N(Trt)C(Trt)AGH(Trt)K(Boc)-OCH$_2$-Wang-resin (P3 peptide).

The deprotected P1 peptide (AAAGGGAAAAK) anchored to the dried resin was stirred with a 20 % piperidine solution at room temperature for 30 minutes, washed with DMF and dried under a vacuum. The peptide-resin was treated with an excess of the non-cleavable heterobifunctional protein cross-linker BMPS ((N-$\beta$-maleimidopropyloxy)succinimide ester) from MERCK dissolved in DMF spiked with traces of trimethylamine and the reaction proceeded during a period of 30 minutes. The resin was washed with DMF and dried under a vacuum. In the case of P4, it was dissolved in 100 mM Tris/HCl buffer, pH=6.8 and treated with an excess of a solution of BMPS in DMF. The reaction proceeded for 30 minutes and the P4 peptide modified with a maleimide group at the $\varepsilon$-amino group of Lysine residue was purified by rp-HPLC.

All peptides anchored to the resin were deprotected and cleaved from the resin by treatment with a mixture containing TFA:H$_2$O:TMS in a ratio of 95:2.5:2.5 (v/v). The peptides were precipitated with diethyl ether, the excess diethyl ether was removed by centrifugation and the peptide pellet was dried under a stream of nitrogen. These peptides were dissolved in H$_2$O/TFA (0.1 % v/v) and purified by rp-HPLC.

As a result, four peptides were synthesized: P1 (*AAAGGGAAAAK), P2 (CAAGGGAAAAK), P3 (NCAGHK), and P4 (Ac-GANAPK*EPQR). Asterisks in the sequences of P1 and P4 peptides mean that their primary amino groups were modified with an N-$\varepsilon$-propionyl maleimide group. Ac- in the P4 peptide sequence indicates that an acetyl group is located at the N-terminal end.

Cross-linked peptides were synthesized by the maleimide-thiol chemistry$^{1,2}$. Approximately equal amounts of the free Cys containing peptides (P2 and P3) and the maleimide N-$\varepsilon$-propionyl maleimide-containing peptides (P1 and P4) were dissolved at equal concentrations in 100 mM Tris/HCl, pH=6.8 and mixed in the correct combinations to synthesize four cross-linked peptides with the thiosuccinimide linker. These compounds are referred to hereafter as (P1-P2), (P1-P3), (P3-P4), and (P2-P4). The reactions proceeded during 20 minutes at room temperature with stirring. The final products of the reactions were analyzed by rp-HPLC and the completion was confirmed by the fact that one of the starting peptides had been completely consumed. The new generated peak fractions that potentially contained the desired product were collected and directly analyzed by MALDI-MS.

Preparation of cross-linked peptides with the linker transformed to thiazine and hydrolyzed thiosuccinimide variants.

Cross-linked peptides were dried in a speed-vac, dissolved in 100 mM Tris-HCl buffer, pH=8.0, and incubated at 37°C for 6 hours to transform them into a thiazine and/or hydrolyzed thiosuccinimide forms that correspond to P3-P4 in Figure S2, P1-P3 in Figure S3, P2-P4 in Figure S4, and P1-P2 in Figure 2. All of the transformed forms were purified by rp-HPLC, collected, and analyzed by MALDI-MS, -MS/MS and LC-MS/MS embedded with UVPD (see below).

RP-HPLC analysis

The peptides were purified with an HP1100 HPLC system (Agilent Technologies, Inc) on a column (ZORBAX Eclipse Plus C18 RRHD 2.1x50 mm, 1.8 μm, Agilent Technologies, Inc) that had been equilibrated in 98 % of solution A (0.1 % v/v TFA in ultrapure water). The concentration of solution B (TFA, 0.1 % v/v in pure ACN) in the mobile phase was increased to 55 % in 15 minutes and reached 80 % of solution B in 0.1 minutes. The column was washed for an additional 0.5 min in 80 % of solution B and was then equilibrated under the initial conditions for a new separation. The flow rate was 0.35 mL/min and the absorbance was monitored at
210 nm during the chromatographic separation. The temperature of the column was maintained at during all chromatographic separations.

MALDI-MS analysis

MALDI-TOF MS was carried out with a 4800 MALDI-TOF/TOF mass spectrometer from Applied Biosystems (Framingham, MA, USA). All mass spectra were obtained by averaging 2500 laser shots from each sample well in the positive-ion mode. The instrument was controlled using the 4000 series Explorer software (version 3.6; Applied Biosystems). Data were processed by using Data Explorer software (version 4.8; Applied Biosystems). The calibration of the instrument in the MS and MS/MS modes were carried out by using the ions derived from a mixture of several synthetic peptides (Angiotensin I, 1296.685; Dynorphin A, 1603.991; ACTH (1-24), 2932.588; beta-endorphin, 3463.830), and several product ions derived from angiotensin I, respectively. Ordinary MS/MS spectra were obtained with a fully automatic workflow, in which the metastable suppressor and CID gas (air) were both activated.

Peptides dissolved in water/acetonitrile/TFA solutions (0.5 μL) were mixed with the (0.5 μL) matrix solution, (the supernatant of a 50 % acetonitrile solution saturated with α-cyano-4-hydroxycinnamic acid), and then air dried on the flat surface of a stainless steel plate. The ions were generated by irradiating the sample area with the output of an Nd/YAG laser at a wavelength of 355 nm, and the resulting generated ions were accelerated at a 20 kV potential in the ion source.

LC-MS/MS with UVPD fragmentation

Peptide fragmentation by UVPD was carried out in an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Data were acquired by the DDA method and Orbitrap detection. MS1 was collected in the mass range from m/z 375 to m/z 1500 at a 120,000 resolution with an AGC target of 1 x 10⁶ and a maximum injection time of 100 ms. Followed by MS1, MS2 was collected at a 30,000 resolution with an AGC target of 1 x 10⁵ and a maximum injection time of 55 ms. Each precursor was isolated with a 2.0 Th window. The peptides were fragmented by laser irradiation in the central part of the quadrupolar dual linear ion trap, and the fragment ions were transferred to the Orbitrap mass analyzer for measurement of their molecular masses. Peptides were photoactivated during 500 ms with the fifth harmonic Q-switched 213 nm Nd:YAG laser (CryLaS, GmbH). Peptides were applied to the Vanquish Neo UHPLC system (Thermo Fisher Scientific, Waltham, MA), and separated on a 75 μm ID x 125 mm capillary column (Nikkyo Technos, Tokyo, Japan) with a linear gradient from 0% to 45% of solvent A/B (A : water with 0.1% formic acid; B : 80% acetonitrile with 0.1% formic acid (Thermo Fisher Scientific, Waltham, MA)) for 10 minutes (synthetic peptides) or 60 minutes (conjugates) at a flow rate of 300 nL/min and sprayed into the mass spectrometer with a 2000 V spray voltage as the ESI.

Results and Discussion:

MALDI-MS analyses of cross-linked peptides with a thiosuccinimide linker and their hydrolyzed variants.

Considering the integrity of structural variants (hydrolyzed and thiazine forms) of the linker moiety, which are relevant to the conjugation modes, four types of cross-linked peptides were prepared (Table 1). P3-P4 and P1-P3 gave only the hydrolyzed forms upon incubation under alkaline conditions (see Materials and Methods). P1-P2 and P2-P4 both gave the hydrolyzed and thiazine forms, the latter of which was the product of transcyclization between the N-terminal Cys of P2 and succinimide (see Figure 1).

The rp-HPLC profile of the P1-P2 cross-linked peptide showed a major fraction at 4.86 min (see the lower chromatogram in Figure 2). MALDI-MS analysis of this fraction (Figure 3a) confirmed the identity of this molecule. Intense signals at m/z 1812.88 and m/z 906.96 were assigned to (M+H)⁺ and (M+2H)²⁺ ions of this cross-linked peptide with the thiosuccinimide linker, respectively (Table 1).

The signals at m/z 847.43 and m/z 968.50 had an appreciable intensity and were assigned to the P2 peptide with a free thiol and the P1 peptide linked to an N-propionyl succinimide, respectively (Figure 3a, structure (II) in 3d), which were confirmed by MS/MS analysis (Figures S5 and S6a) and were most likely produced
via reductive cleavage during the MALDI procedure. In the MALDI-MS analysis of the crosslinked peptides prepared with other crosslinker reagents based on similar maleimide-thiol chemistry, signals resulting from cleavage of the thioether bond were also observed\textsuperscript{19,20}.

Note that an expanded region of this MALDI-MS spectrum (see inset Figure 3a) also showed the presence of an ion at $m/z$ 966.49, which corresponds to the P1 peptide, but with an $N$-propionyl maleimide group (structure (I) in Figure 3d). This fragment ion could be generated via a 1,4-elimination reaction at the thioether bond as depicted in Figure 4a, which was also observed by MS/MS analysis (Figure S6b).

The P1-P2 peptide but with a hydrolyzed linker, referred to as (P1-P2)$_H$ (rt. 4.70 min in the upper chromatogram, Figure 2), was prepared by incubating the original P1-P2 peptide (rt. 4.86 min in Figure 2, lower chromatogram) at pH=8.0 for 6 hours at 37°C. Its molecular mass was observed at $m/z$1830.64 (Figure 3b), the mass of which was larger by 18 Da than the original one (Figure 3a), suggesting that either of the two imide bonds in the thiosuccinimide had been hydrolyzed by the hydrolytic process (see structure (I) in Figure 1). It is noteworthy that the signals at $m/z$ 847.32, 984.36 and 986.38 in Figure 3b could be assigned to the P2 peptide with a thiol and the P1 peptide with the counterpart but with the hydrolyzed linker (structure (III) and (IV) in Figure 3d), which were likely produced via 1,4-elimination or reductive cleavage, respectively, as observed for (P1-P2).

The above results clearly suggest that the linkage between the succinimide ring and Cys could be cleavable upon MALDI, the fact of which is also supported by the results obtained for the other cross-linked peptides, three of them with the thiosuccinimide linker (P1-P3), (P2-P4) and (P3-P4), and their corresponding analogs with a hydrolyzed thiosuccinimide linker, (P1-P3)$_H$, (P2-P4)$_H$ and (P3-P4)$_H$ (see Figures S7-S9).

Considering the strong absorption of laser energy by hot matrices such as $\alpha$-CHCA in MALDI and the subsequent transfer of energy to the analyte, it can be assumed that the excess energy stored in the analyte ions led to the spontaneous dissociation of the thioether bond, which is a relatively weak chemical bond. This result is consistent with the MALDI ionization method and suggests that thioether-based linkers are cleavable when the MALDI ionization method is used in such an analysis, and consequently provides useful information concerning the components of the cross-linked peptide.

Differentiation by MALDI-MS and -MS/MS analysis of cross-linked peptides with thiosuccinimide and thiazine linkers.

The MALDI-MS spectra of cross-linked peptides (P1-P2) and (P2-P4) with a thiosuccinimide linker were compared with their corresponding isomers with a thiazine linker. Both of the cross-linked peptides with the thiosuccinimide linker gave a signal at $m/z$ 847 which corresponds to the thiol-containing peptide (P2-SH). Their counterparts, linked to the $N$-propionyl maleimide or $N$-propionyl succinimide, were observed at $m/z$ 966.49 and 968.50 (inset of Figure 3a), and $m/z$ 1260.32 and 1262.33 (inset of Figure 5a), which were assigned to P1-(I) and P1-(II) in Figure 3d, and P4-(I) and P4-(II) in Figure 5c, respectively. As in the case of P1* in the (P1-P2) cross-linked peptide (see Figure 3a), the P4 peptide was observed as $N$-propionyl maleimide or $N$-propionyl succinimide forms (Figure 5c), which arose from 1,4-elimination or reductive cleavage, respectively, at the C-S linkage between the Cys residue and the succinimide during the MALDI process.

The cross-linked peptides, P1-P2 and P2-P4, gave significant amounts of the isobaric thiazine forms under coupling conditions (Figure 2 and Figure S4) and are denoted as (P1-P2)$_T$ and (P2-P4)$_T$. As described above, this transformation from the thiosuccinimide linker predominantly occurs in a spontaneous manner for the case where a conjugated peptide contains an $N$-terminal Cys. The molecular mass of the thiazine form was the same as that of the original thiosuccinimide form, but none of the fragment ions characteristic of the peptide with a thiosuccinimide linker were observed (Figures 3c and 5b). This result suggests that the thiazine linker is stable and resistant to cleavage during MALDI while the thiosuccinimide linker or its hydrolysis form is amenable to cleavage at the C-S linkage of the linker (Figures 3a and 5a).

These results suggest that the thiosuccinimide forms, including those that had been hydrolyzed, can be
unambiguously identified by observing the peptides constituting the conjugate simultaneously in MALDI-MS (see Figures 3a, 3b, 5a, and S7-S9). In contrast, the thiazine form does not yield these peptide ions (Figures 3c and 5b). This suggests that the thioether bond, embedded in the six-membered ring of thiazine, is not susceptible to such cleavage of the conjugate that yields the two constituent peptides.

MALDI-MS/MS was then examined in an attempt to distinguish these isobaric peptides. The MALDI-MS/MS spectra of (P1-P2) with thiosuccinimide and (P1-P2)T with the thiazine linker were nearly identical, with respect to the m/z values of the backbone fragment ions for the P1 and P2 peptides (Figures 6a and 6d, Figures S10 and S11). However, there was a marked difference in the expanded region (m/z 792-1013, Figures 6b and 6c). The thiosuccinimide form (P1-P2) yielded two pairs of complementary fragment ions that were produced via a 1,4-elimination: Cys-SH and maleimide (Figure 4a), and dehydro-alanine and thiosuccinimide (Figure 4b), which was distinctly different from those produced via reductive cleavage in MALDI-MS (see Figure 3a). The other cross-linked peptides, (P1-P3) and (P3-P4), with the thiosuccinimide linker produced fragment ions that were similar to those observed for (P1-P2) (see Figures S12 and S13).

In the case of (P1-P2)T, the thiazine form, which is formed primarily when a cysteinyl peptide is cross-linked to another peptide containing Lys by BMPS (upper chromatogram in Figure 2, retention time 5.10 min), no ions were observed when the above C-S bond was cleaved. Instead, a pair of ions at m/z 886.36 (P1+71) and its counterpart of ion at m/z 927.30 (P2+L-71, where L denotes ‘linker’), highlighted in red, were newly observed (Figures 6c and 6d). These ions, specifically observed for (P1-P2)T, were those produced when cleaved at the new pseudo-peptide bond generated in the thiazine-containing linker (Figure 1, structure (IV)). Similar results were also obtained for (P2-P4)T (see Figures S14 and S15).

The above results demonstrate that MALDI-MS/MS allowed both isobaric cross-linked peptides with thiosuccinimide and thiazine linkers to be clearly differentiated, based on the fragment ions produced via a 1,4-elimination at the thiosuccinimide/Cys residue and the fragmentation of the newly formed pseudopeptide bond in a thiazine linker, respectively, the latter of which has also been observed by LC-MS/MS using HCD as a fragmentation method 15.

UVPD fragmentation of cross-linked peptides with thiosuccinimide and thiazine linkers

Ultraviolet photodissociation (UVPD) has been applied for the identification of disulfide heterogeneity and non-native crosslinks such as trisulfide and thioether bonds which are generated during the production of and storage of monoclonal antibodies 21. UVPD at 213 nm can be used to selectively dissociate these bonds to produce characteristic fragmentation patterns which are derived from the homolytic cleavage of S-S and C-S linkages 22. Based on these previous observations, we examined the issue of whether or not conjugates containing thiosuccinimide or thiazine linkers could be distinguished by the UVPD method incorporated in LC/ESI-MS/MS (see Experimental section). For P2-P4, product ions at m/z 813.42, 847.41 and 1260.60 in Figure S16a, observed for the thiosuccinimide form, were similar to those observed in MALDI-MS/MS (Figure S14), which most likely had arisen from a 1, 4-elimination (Figure 4). Note that the observed product ions were not those derived from homolytic cleavage at the C-S linkage of the thiosuccinimide, since they were smaller by 1 Da than those from the homolytic cleavage. These ions were sufficient for identifying the thiosuccinimide form, whereas, UVPD-MS/MS of the thiazine form did not give any product ions derived from the cleavage at the pseudopeptide bond which was newly formed in the thiazine form (Figure S16b). Considering the conditions of the UVPD used in this study, i.e., photoactivation time (500 ms) and the method of ion trapping in the device, it is likely that the precursor ions primarily underwent spontaneous fragmentation by 1, 4- elimination rather than dissociation by UV-laser irradiation.

Conclusions:

MALDI-MS analysis can be used to distinguish isobaric cross-linked peptides with thiazine and thiosuccinimide linkers. Peptides that are cross-linked with thiazine linkers are very stable in MALDI and are thus detected only as (M+H)+ ions. On the other hand, cross-linked peptides with thiosuccinimide as the linker are clearly more susceptible to degradation by MALDI, and the thioether bond of thiosuccinimide is partially cleaved. As a result, in addition to the intact cross-linked peptides, the free thiol-containing peptides and
peptides attached to the original N-propionyl succinimide, which constitute the conjugate, are clearly detected in the MALDI-MS spectra. This behavior was also observed for cross-linked peptides with hydrolyzed thiosuccinimide linkers.

MALDI-MS/MS analysis can more reliably distinguish isobaric cross-linked peptides with thiazine and thiosuccinimide as linkers. The former gives fragment ions derived from cleavage at the new pseudo-peptide bond produced by trans-cyclization in the thiosuccinimide linker; the latter gives two types of the fragment ions due to 1,4-elimination that occurs in the gas phase (Figure 4). MALDI-MS or MALDI-MS/MS can be used as a simple technique to identify isobaric cross-linked peptides that are prepared via maleimide chemistry.

Figures Legends:

Figure 1. Possible cross-linking structures between Cys and Lys prepared by thiol-maleimide chemistry. Structure (I): thiosuccinimide form; (II), (III): Hydrolyzed forms of (I), in which the five-membered thiosuccinimide ring was cleaved at “a” or “b”, respectively; (IV): thiazine form, which is formed by the attack of the α-amino group of Cys on the carbonyl of the succinimide moiety and has the same molecular mass as (I). The dashed square in Structure IV shows the new pseudo-peptide bond present in the thiazine ring.

Figure 2. Reverse-phase liquid chromatograms of the P1-P2 cross-linked peptide obtained after the synthesis (b) and incubation in 100 mM Tris/HCl buffer, pH=8.0 for 6 hours at 37°C (a). The amino acid sequences of P1 and P2 are (A AAGGGAAAAK) and (C AAGGGAAAAK), respectively. The peaks marked with asterisks in both chromatograms correspond to the disulfide-linked P2-S-S-P2 homodimer formed as a by-product. The left and right Y-axes represent the absorbance (mV) at 210 nm of the upper and lower chromatograms, respectively. Numbers in red above each fraction indicate retention times.

Figure 3. MALDI-MS spectra of the cross-linked peptide, P1 (A AAGGGAAAAK)-P2 (C AAGGGAAAAK) (a), its hydrolyzed (P1-P2)H (b) and thiazine (P1-P2)T (d) forms. The insets in (a) and (b) are the expanded views of the red dashed squares. The signals marked with I, II, III, and IV in the insets are assigned to the structures in (d). The structures of P1-P2, (P1-P2)H and (P1-P2)T are depicted at the right side in (a), (b) and (c), respectively. P2-SH in the spectra correspond to the P2 peptide with a free Cys residue.

Figure 4. The 1,4-elimination observed for the cross-linked peptide containing thiosuccinimide. The two possible pathways are depicted in (a) and (b). Pn denotes the peptides used in this study.

Figure 5. MALDI-MS spectra of the cross-linked peptides, P2 (C AAGGGAAAAK)-P4 (Ac-GANAPK EPQR) (a) and the thiazine form (P2-P4)T (b). The inset of (a) is the expanded view of the red dashed square. P2-SH corresponds to the P2 peptide with a free Cys residue. The signals marked with P4* in (a) correspond to the structures depicted in (c).

Figure 6. MALDI-MS/MS spectra of the cross-linked peptides, P1-P2 (a) and (P1-P2)T (d). The areas enclosed by the dashed squares in (a) and (d) are enlarged in (b) and (c), respectively. The m/z values in green and red indicate fragment ions that are specifically observed for the thiosuccinimide and thiazine forms, respectively. “L” indicates the N-propionyl maleimide linker. The assignments of backbone-derived fragment ions are summarized in Figures S10-S11.

References:


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