Mapping Protein Interfaces by a Trifunctional Cross-Linker Combined with MALDI-TOF and ESI-FTICR Mass Spectrometry

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Chemical cross-linking of protein complexes has gained renewed interest in combination with mass spectrometric analysis of the reaction products as it allows a rapid mapping of protein interfaces, which is crucial for understanding protein/protein interactions. The identification of cross-linking products from the complex mixtures created after the cross-linking reaction, however, remains a daunting task. To facilitate the identification of cross-linking products, we explore the use of the commercially available biotinylated cross-linking reagent sulfo-SBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-hexanoamido]ethyl-1,3′-dithiopropionate). This trifunctional cross-linker possesses one amine-reactive and one photoreactive site and, additionally, allows an affinity-based enrichment of cross-linker containing species. As a model system, we chose the Ca^{2+}-dependent complex between calmodulin and its target peptide M13, which represents a part of the C-terminal sequence of the skeletal muscle myosin light chain kinase. After the cross-linking reaction, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and one-dimensional gel electrophoresis were employed to check for the extent of cross-linking product formation. The cross-linking reaction mixtures were subjected to tryptic *in-solution* digestion. Biotinylated peptides, e.g., peptides that had been modified by the cross-linker as well as cross-linked peptides, were enriched on monomeric avidin beads after several washing steps had been performed. Peptide mixtures were analyzed by MALDI-TOFMS, nano-high-performance liquid chromatography (HPLC)/nano-electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICRMS), and tandem MS. We demonstrate that an enrichment of cross-linker containing species allows a more efficient identification of interacting amino acid sequences in protein complexes. This strategy is expected to be especially beneficial for investigating large protein assemblies. (J Am Soc Mass Spectrom 2005, 16, 1921–1931) © 2005 American Society for Mass Spectrometry

A detailed knowledge of interacting amino acid sequences in protein complexes is of outstanding importance for understanding protein functions in a cell. A number of well-established analytical techniques are available to address the questions of spatial and topological organizations of protein complexes, currently primarily X-ray crystallography and NMR spectroscopy. Both techniques yield detailed information on the protein structure, however, NMR spectroscopy requires rather large quantities of pure protein in a specific solvent, and for X-ray studies the protein has to be crystallized. A large-scale analysis of proteins in the post-genomic research requires sensitive techniques for high-throughput analysis such as mass spectrometry. Among the mass spectrometric techniques used for structural studies of proteins are hydrogen/deuterium exchange [1], analysis of the intact noncovalent complexes in the gas-phase [2], and chemical cross-linking followed by enzymatic digestion and mass spectrometric identification of the cross-linked peptides [3, 4]. Mass spectrometry [5, 6] is the method of choice for the latter studies because of its high sensitivity, enabling rapid analysis of the complex mixtures obtained from enzymatic digests of cross-linking reaction mixtures [7, 8]. Chemical cross-linking combined with mass spectrometry represents a low-resolution alternative to NMR spectroscopy and X-ray crystallography-based methods. However, despite the apparent straightforwardness of the cross-linking approach, cross-linking has always been a trial and error process for a particular protein complex, and identification of cross-linking products is far too often hampered by the complexity of the cross-linking reaction mixture. To overcome these difficulties, a number of strategies have been employed to facilitate detection of the cross-linking products, such as using isotope-labeled cross-linkers or proteins, fluorogenic cross-linkers, or cross-linkers creating charac-

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teristic fragment ions during mass spectrometric detection [4]. A relatively new group of reagents are trifunctional cross-linkers, such as the cross-linker sulfo-SBED which was used for the present study, possessing three different reactive groups. The trifunctional approach incorporates elements of the heterobifunctional cross-linker concept with the additional third functional group being able to specifically link to a third protein or being used for affinity purification of the cross-linking products in case a biotin moiety is incorporated [9].

In the present study, we explore the possibility that a combination of chemical cross-linking using the affinity cross-linker sulfo-SBED and FTICR (Fourier transform ion cyclotron resonance) mass spectrometry allows an efficient identification of interacting amino acid sequences in protein complexes, as cross-linker containing species are enriched by an affinity purification step after the cross-linking reaction. We apply this strategy to the complex between the 17 kDa-protein calmodulin (CaM) and its target peptide M13 from the skeletal muscle myosin light chain kinase, which has already been studied by our group using chemical cross-linking and FTICR mass spectrometry [10].

CaM is a small (148 amino acids), acidic protein belonging to the class of EF-hand proteins, which is found ubiquitously in animals, plants, fungi, and protozoa [11]. CaM serves as a calcium-dependent regulator in many metabolic pathways [12]. Upon calcium binding, CaM adopts a dumbbell structure [13] consisting of two lobes connected by a flexible central helix [14]. CaM is known to bind with high affinity to various target proteins and peptides, with dissociation constants ranging between 10^{-7} and 10^{-11} M [11].

The myosin light chain kinase of the skeletal muscle (skMLCK) belongs to a class of CaM-dependent enzymes consisting of ca. 600 amino acids depending on the species. Binding studies using chymotryptic fragments of the rabbit skMLCK and synthetic peptides identified a C-terminal peptide of the skMLCK as the CaM-binding region [15]. This 26-amino acid peptide has been designated as M13, comprising amino acids 577-602 of the rabbit skMLCK (or amino acids 565–590 of the human skMLCK, respectively). Accordingly, M13 represents the complete CaM-interacting sequence of skMLCK and, thus, can be employed as a substitute for the total kinase to analyze the structures of CaM and the interacting skMLCK-sequence within the complex [16]. The three-dimensional structure of the CaM/M13 complex was solved several years ago by multi-dimensional NMR spectroscopy [16] and, moreover, we had also studied this complex by chemical cross-linking and FTICR mass spectrometry [10] making this complex an ideal system to test our method.

Recently, Hurst et al. demonstrated the application of the cross-linker sulfo-SBED in combination with MALDI-TOF mass spectrometry for identification of intramolecular cross-linking products and cross-linker modified amino acids within the 13-amino acid model peptide neurotensin [17]. Sulfo-SBED had also been employed to identify an interacting region between α-crystallin and alcohol dehydrogenase [18].° To° our knowledge, the present study is the first report on an affinity purification of cross-linker-containing, biotinylated species in combination with MALDI-TOFMS and ESI-FTICRMS. The reported strategy presents a straightforward approach with the perspective to obtain structural data of protein complexes within a few days.

Experimental

Material

Bovine brain CaM was obtained from Calbiochem (Schwalbach am Taunus, Germany) and used without further purification. The skMLCK peptide (M13) was a generous gift from Dr. Ad Bax, National Institutes of Health, U.S.A. The purities of CaM and M13 were checked by MALDI-TOF mass spectrometry and one-dimensional gel electrophoresis (SDS-PAGE). The cross-linking reagent sulfo-SBED (sulfosuccinimidyl-2-[6-(biotin-amido)-2-(p-azidobenzamido)-hexanoamido]-ethyl-1,3′-dithiopropionate) and UltraLink immobilized monomeric avidin were purchased from Pierce (Rockford, IL). Trypsin (sequencing grade) was obtained from Roche Diagnostics (Mannheim, Germany). LHRH (luteinizing hormone releasing hormone), 2,5-dihydroxy benzoic acid, α-cyano-4-hydroxycinnamic acid, as well as proteins and peptides for MALDI-TOFMS calibration were purchased from Sigma (Taufkirchen, Germany). Buffer reagents were obtained from Sigma (Taufkirchen, Germany) at the highest available purity. Nano-HPLC solvents were spectroscopic grade (Uvasol, VWR, Darmstadt, Germany). Water was purified with a Direct-Q5 water purification system (Millipore, Eschborn, Germany).

Cross-Linking Reactions

For cross-linking experiments, an aqueous CaM stock solution (1 mg/ml) was diluted with 20 mM HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$ to give three solutions with volumes of 1400 μl each, containing CaM at a concentration of 10 μM.

To each CaM solution, a 100 mM solution of sulfo-SBED in DMSO was added, yielding molar excesses of cross-linker over protein/peptide of 20, 50, and 100, respectively. The mixtures were incubated for 60 min at room temperature. Excess of nonreacted cross-linker was removed using Microcon-YM-10 filters (Millipore, Eschborn, Germany) and the three samples (20-, 50-, and 100-fold excess of sulfo-SBED) were recovered in 3 × 1400 μl of 20 mM HEPES buffer (pH 7.0) containing 1 mM $CaCl_2$. One 200-μl aliquot was taken from each sample and stored at −20 °C before SDS-PAGE and MALDI-TOFMS.

A 330 μM aqueous solution of M13 was added to the three reaction mixtures to give a final M13 concentra-

tion of 10 μM. The samples were irradiated with long wavelength UV light (365 nm) using a MinUvis lamp (8 watt, Desaga, Heidelberg, Germany). During irradiation, the reaction mixtures were kept at 0 °C in an ice bath. Two hundred-μl aliquots were taken after 15 min, 30 min, 60 min, 120 min, 8 h, and 16 h. The reactions were terminated by freezing the samples at −20 °C. Before SDS-PAGE and MALDI-TOFMS, the solutions were desalted using Microcon-YM-10 filters (Millipore, Eschborn, Germany). As, in contrast to previously conducted°experiments°with°sulfo-SBED°[17],°we°did°not aim to cleave the disulfide bond of the cross-linker, there was a need to maintain nonreducing conditions during all stages of the analysis.

SDS-PAGE and Enzymatic Proteolysis

SDS-PAGE°(15%,°Coomassie°staining,°[19]°under°nonreducing conditions was employed to check for the extent of cross-linking product formation in the reaction mixtures. To 100 μl of each aliquot, 8M urea was added and *in-solution* digestion was performed by adding 0.25 μg trypsin (corresponding to an enzyme/substrate ratio of ~1:80) followed by incubation at 37 °C for 16 h before the samples were subjected to affinity purification on avidin beads.

Affinity Purification of Cross-Linker Containing Species

Avidin affinity capture of biotinylated species was performed using immobilized monomeric avidin (Pierce Inc., Rockford, IL). The avidin beads in 50% aqueous suspension were prepared for affinity purification of biotinylated species in the following way: the beads were successively washed with 50 mM sodium phosphate buffer (pH 7.0), 100 mM glycine buffer (pH 2.8), 50 mM sodium phosphate buffer (pH 7.0), 2 mM biotin in 100 mM HEPES buffer (pH 7.5), 100 mM glycine buffer, (pH 2.8) 15% methanol in 50 mM NH_4HCO_3, and 50 mM sodium phosphate buffer (pH 7.0). As a last step, the same volume of water was added to the beads and 50 μl of peptide digest was added to 140 μl of the avidin beads suspension. The mixtures were incubated at room temperature in the dark for 3 h, centrifuged, and the supernatants were collected. The avidin beads were washed three times with 400 μl of 50 mM sodium phosphate buffer (pH 7.0) and twice with 15% methanol in 50 mM NH_4HCO_3. Biotinylated peptides, thus cross-linker containing species, were eluted from the beads with a 50% aqueous acetonitrile solution containing 0.4% formic acid. The fractions (one supernatant, three phosphate fractions, two methanol fractions, and two acetonitrile fractions from each sample) were brought to complete dryness in a vacuum concentrator (Eppendorf, Hamburg, Germany). The supernatants and the phosphate fractions were desalted using C18 ZipTips (Millipore, Eschborn, Germany). All samples were collected in 30 μl of 0.1% formic acid before mass spectrometric analysis by MALDI-TOFMS and nano-HPLC/nano-ESI-FTICRMS was performed.

MALDI-TOF Mass Spectrometry

MALDI-TOFMS was performed on a Voyager DE RP Biospectrometry Workstation (Applied Biosystems, Foster City, CA) equipped with a nitrogen laser (337 nm). The instrument was run in positive ionization mode and measurements were performed in the linear mode (*m/z* 15,000 to 40,000) as well as in the reflector mode (*m/z* 1000 to 6000) using 2,5-dihydroxy benzoic acid and α-cyano-4-hydroxycinnamic acid (HCCA) as matrices. A saturated matrix solution was prepared in 30% (vol/vol) acetonitrile, 69.9% (vol/vol) water, and 0.1% (vol/vol) TFA. Samples were prepared using the dried droplet method by spotting 0.5 μl of matrix solution and 0.5 μl of sample solution onto the target. Spectra from 800 laser shots were accumulated to one spectrum when operating the instrument in the reflector mode, and 1500 laser shots were added when spectra were acquired in the linear mode. In the linear mode, the instrument was calibrated using cytochrome *c* ($[M + H]^+_{average}$ at *m/z* 12,361) and myoglobin ($[M + H]^+_{average}$ at *m/z* 16,952). For calibration in the reflector mode, signals of angiotensin I ($[M + H]^+_{mono}$ at *m/z* 1296.69), substance P ($[M + H]^+_{mono}$ at *m/z* 1347.74), and somatostatin (reduced form) ($[M + H]^+_{mono}$ at *m/z* 1637.72) were employed. Data acquisition and data processing were performed using the Voyager software version 5.1 and the Data Explorer software version 4.0 (Applied Biosystems, Foster City, CA).

Nano-HPLC/Nano-ESI-FTICR Mass Spectrometry

Enzymatic peptide mixtures from various stages of the avidin separation were separated by nano-HPLC. Nano-HPLC was carried out on an Ultimate Nano-LC system (Dionex, Idstein, Germany) equipped with a Switchos II column switching module and a Famos Micro Autosampler with a 20-μl-sample loop. Samples were injected by the autosampler and concentrated on a trapping column (PepMap, C18, 300 μm × 5 mm, 3 μm, 100 Å, Dionex) with water containing 0.1% formic acid at flow rates of 20 μl/min. After 3 min for desalting, the peptides were eluted onto the separation column (PepMap, C18, 75 μm × 150 mm, 3 μm, 100 Å, Dionex), which had been equilibrated with 95% A (Solvent A: water containing 0.1% formic acid). Peptides were separated using the following gradient: 0–30 min: 5–50% B, 30–31 min: 50–95% B, 31–45 min: 95% B (Solvent B: acetonitrile containing 0.1% formic acid) at flow rates of 200 nl/min and detected based on their UV absorptions at 214 and 280 nm. For LC/MS data acquisition, the Hystar software, version 2.3 (Bruker Daltonics, Billerica, MA) was used.

The nano-HPLC system was coupled *on-line* to an FTICR mass spectrometer equipped with a 7 tesla

superconducting magnet (APEX II, Bruker Daltonics) and a nano-electrospray ionization source (Agilent Technologies, Waldbronn, Germany). For nano-ESIMS, distal coated fused-silica PicoTips (tip ID 8 μm, New Objective, Woburn, MA) were applied. The capillary voltage was set to −1400 V. Mass spectral data were acquired in the broadband mode over an *m/z* range of 400 to 2000 with 256 k data points, 10 scans were accumulated per spectrum, and 162 spectra were recorded for each LC/MS run. MS data acquisition was initialized from the Hystar software 10 min after initiation of the LC gradient. Data were acquired over 34.5 min. Calibration of the instrument was performed with CID fragments (capillary exit voltage 250 V) of the LHRH peptide (y5 (*m/z* 499.2987), LHRH ($[M+H]^{2+}$ *m/z* 592.2358), y7 (*m/z* 749.3941), y8 (*m/z* 935.4734), LHRH ($[M+H]^{+}$ *m/z* 1183.5643)). Data acquisition and data processing were performed using the XMASS software, version 7.0.3 (Bruker Daltonics). Before Fourier transformation the time-domain signals were doubly zero filled, followed by apodization with a sine function. 162 single spectra were projected into one final mass spectrum using the '*Projection*' tool in the XMASS software [20]. After deconvolution of the ESI mass spectra, monoisotopic peptide masses ($[M+H]^{+}$) were manually labeled and the created peak lists were used for calculating masses of cross-linking products by the GPMAW software.

Nano-ESI-MS/MS Analysis

MS/MS data of a fraction eluted from the avidin beads with 50% aqueous acetonitrile and 0.4% formic acid were acquired using a linear ion trap mass spectrometer (Finnigan LTQ, Thermo Electron Corp., Waltham, MA, USA) equipped with a nanospray source (PicoView 550, New Objective). The sample was loaded onto a combined reversed-phase column / spray emitter (PicoFrit, BioBasic C-8, 75 μm x 10 cm, tip-ID: 15 μm, New Objective) using a 1-μl-sample loop. Peptides were eluted with the following gradient: 90% A for 10 min, 90% A to 80% B in 15 min, 80 to 95% B in 10 min, 95% B for 10 min (solvent A: H_2O + 0.1% formic acid, solvent B: acetonitrile + 0.1% formic acid) at a flow rate of 300 μl/min. Gradient and solvent flow rate were generated using the Surveyor MS pump (Thermo Electron Corp., Waltham, MA, USA) with a 1:1000 flow split. Eluted peptides were sprayed directly from the PicoFrit column into the mass spectrometer by applying a spray voltage of 1.4 kV.

MS-data were acquired over 45 min, each full MS scan (*m/z* 500–2000) was followed by one zoom scan of the most intense signal in the full MS spectrum to define its charge state and one MS/MS scan (isolation window 2 u) of the same species. Dynamic exclusion was enabled to allow detection of less abundant ions. The LTQ mass spectrometer and the Surveyor MS pump were controlled by the Xcalibur software version 1.4 (Thermo Electron Corp., Waltham, MA).

(a)

^{1}ADQLT	^{6}EEQIA	^{11}EFKEA	^{16}FSLFD	^{21}KDGDG	^{26}TITTK
^{31}ELGTV	^{36}MRSLG	^{41}QNPTE	^{46}AELQD	^{51}MINEV	^{56}DADGN
^{61}GTIDF	^{66}PEFLT	^{71}MMARK	^{76}MKDTD	^{81}SEEEI	^{86}REAFR
^{91}VFDKD	^{96}GNGYI	^{101}SAAEL	^{106}RHVMT	^{111}NLGEK	^{116}LTDEE
^{121}VDEMI	^{126}READI	^{131}DGDGQ	^{136}VNYEE	^{141}FVQMM	^{146}TAK

(b)

^{1}KRRWK ^{6}KNFIA ^{11}VSAAN ^{16}RFKKI ^{21}SSSGA ^{26}L

Figure 1. Amino acid sequences of (**a**) CaM and (**b**) M13. CaM was found to be N-acetylated (Ac-Ala) and to contain a trimethylated lysine in position 115. The modified amino acids Ala-1 and Lys-115 are underlined.

Identification of Reaction Products

Cross-linking products and modified peptides were identified using the General Protein Mass Analysis for Windows (GPMAW) software, version 6.20 (Lighthouse Data, Odense, Denmark) (available at: http://welcome.to/gpmaw). Proteolytic cleavages at modified amino acids, such as the trimethylated K115 in CaM as well as amino acids modified by cross-linking reagents, were excluded. The N-terminus of CaM was excluded from possible cross-linking since it is acetylated.

Determination of Distances Between Atoms in the CaM/M13 Complex

The NMR structure of the CaM/M13 complex is deposited in the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) under the entry 2BBM [16]. Based on the atom coordinates of this structure, the distances between atoms of reactive groups were determined using the VMD-XPLOR visualization package [21] (available at: http://vmd-xplor.cit.nih.gov).

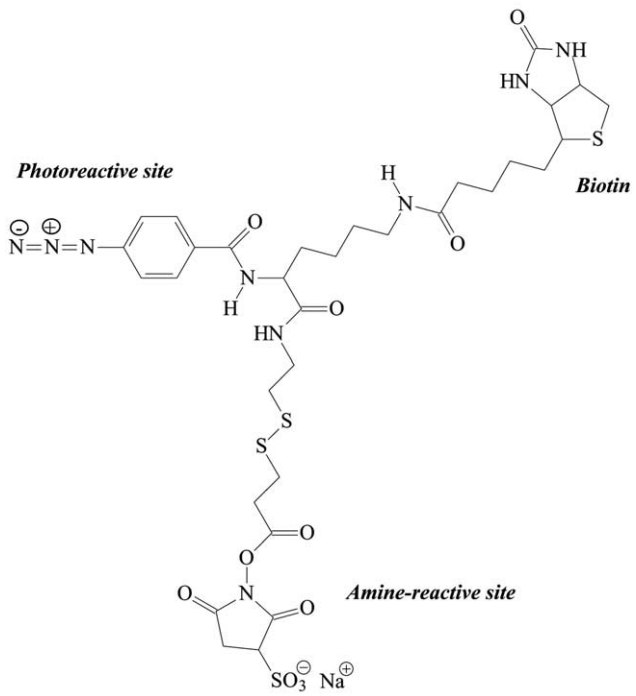
Results and Discussion

Characterization of CaM and M13

The basis for conducting cross-linking experiments with CaM and M13 consists in a detailed description of their respective structures, which are shown in Figure 1. The characterization of both CaM and M13 has been described elsewhere [10].

Cross-Linking Reaction

Scheme **1** shows the structure of the trifunctional cross-linker sulfo-SBED possessing one photo-reactive and one amine-reactive site in addition to a biotin moiety, thus allowing for an affinity purification of cross-linker containing species. Possible reaction products between sulfo-SBED and the protein/peptide, which were considered in this study, are presented in Table 1. Reaction

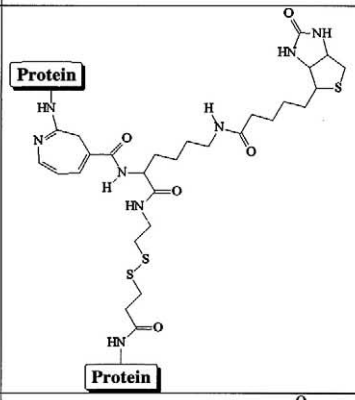
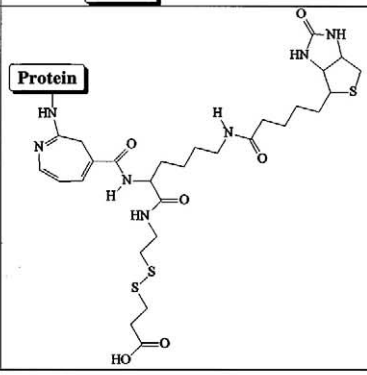
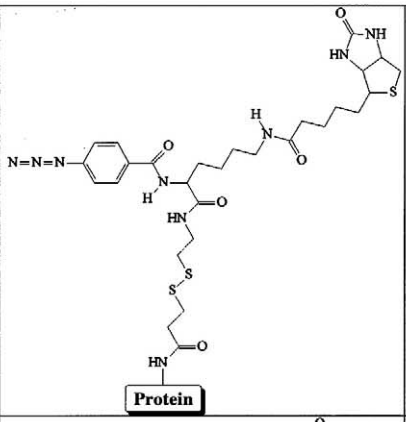
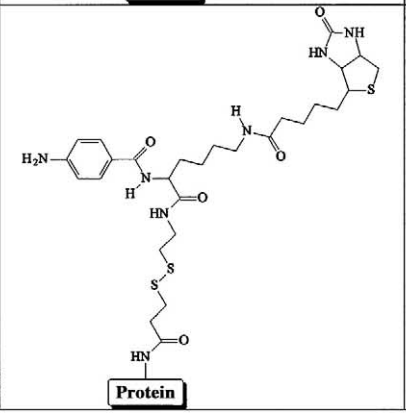


Scheme 1. Structure of the trifunctional cross-linker sulfo-SBED.

products include cross-linked species in which both the N-hydroxy succinimide (NHS) group and the azide group of sulfo-SBED have reacted with the protein/peptide, resulting in the formation of intramolecular (between parts of one protein) and/or intermolecular (between the different complex constituting components) cross-linking products [4]. Moreover, modifications of the protein/peptide are created either by reaction of the photoreactive aryl azide group or by reaction of the NHS-ester with lysine groups or the free N-terminus (Table 1). In the latter case, the azide group is converted to an amine group or, alternatively, stays intact. Oxidized analogs of sulfur-containing species are also occurring quite frequently, which had already been perceived by the studies of Hurst et al. [17]. Therefore, we chose a low-intensity UV lamp for conducting the photoreaction to reduce unwanted oxidation reactions in the protein as much as possible. Moreover, we did not cleave the cross-linker with a reducing agent, such as dithiothreitol (DTT) as we employed FTICR mass spectrometry [22], which allows acquisition of MS data with unprecedented mass accuracy, thus yielding an additional constraint for correct assignment of cross-linker containing species.

The cross-linking reaction conditions in respect to protein/peptide concentration, buffer system, buffer concentration, and pH value had already been optimized in our previous cross-linking experiments with the CaM/M13 system [10] and were employed for the present experiments with sulfo-SBED. The only difference to our previously conducted cross-linking studies is that cross-linking experiments were carried out in a

Table 1. Sulfo-SBED reaction products with their respective mass increases (Δm). For the photoreactive site of sulfo-SBED, a possible reaction product is depicted for a protein amine group, but reaction also occurs with CH-groups

Abbreviation	*Structure*	*Mass Increase Δm*
SBED Cross-Link		634.207 u
SBED A		652.217 u
SBED B-N_3		662.213 u
SBED B-NH_2		636.222 u

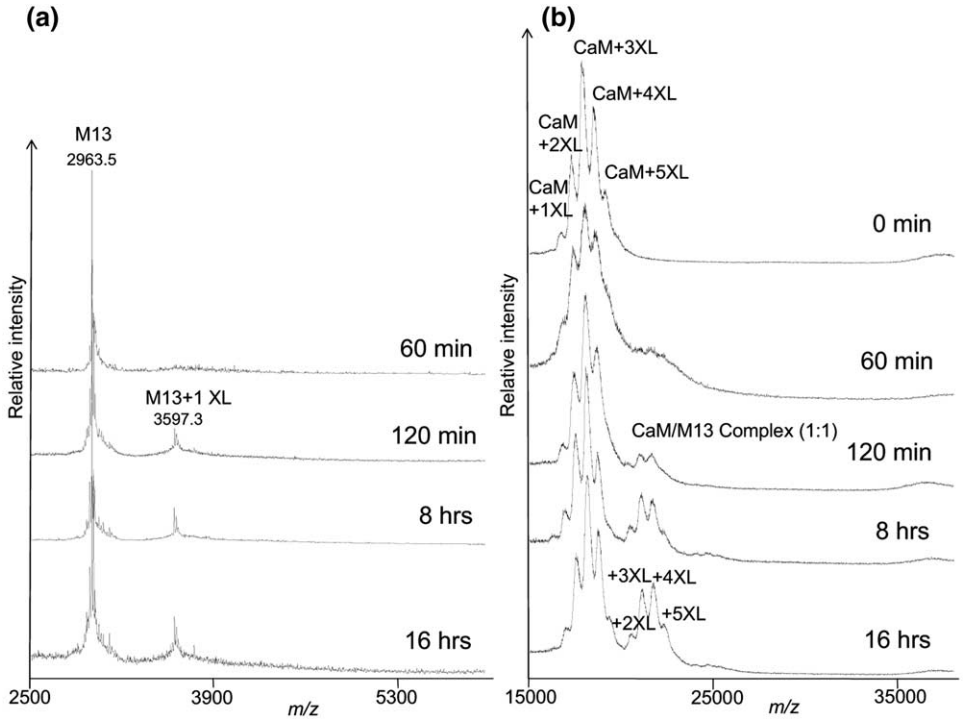


Figure 2. MALDI-TOF mass spectra of reaction mixture between CaM and M13 with 100-fold excess of sulfo-SBED at increasing UV irradiation times (0 min to 16 h). Please note that the sample at 0 min irradiation time was prepared before adding M13 to the reaction mixture. (**a**) MALDI-TOF mass spectra (reflector mode, *m/z* 2500–6000) showing increased modification of M13 by sulfo-SBED with longer UV irradiation times; (**b**) MALDI-TOF mass spectra (linear mode, *m/z* 15,000–40,000) showing increased modification of CaM by sulfo-SBED as well as increased formation of a CaM/M13 (1:1) complex with longer UV irradiation times. XL: cross-linker.

two-stage fashion, in which calcium-loaded CaM was reacted with sulfo-SBED first. After 60 min, excess of sulfo-SBED was removed, M13 was added, and the reaction mixtures were irradiated with long-wavelength UV (365 nm) for varying times between 10 min and 16 h. As cross-linking conducted with a 100-fold excess of sulfo-SBED over the protein/peptide concentration gave the highest yield of cross-linking products (according to SDS-PAGE), we used this fixed concentration of sulfo-SBED (100-fold excess) for further analyses of cross-linking products.

Figure 2 shows MALDI-TOF mass spectra of the cross-linking reaction mixtures recorded in the reflector- (Figure 2a) and in the linear mode (Figure 2b), which allowed for estimating the extent of CaM and M13 modification as well as of cross-linking product formation between both binding partners. Additionally, we conducted SDS-PAGE for reaction control (data not shown). When conducting cross-linking experiments, one has to take care that only a few cross-linker molecules are introduced into the molecule as, otherwise, there might be a distortion of the three-dimensional structures of the proteins by excessive cross-linking.

In the MALDI-TOF mass spectra recorded in the reflector mode, a signal at *m/z* 2963.5 corresponds to the monoisotopic $[M + H]^+$ peak of M13, whereas a signal at *m/z* 3597.3 was assigned to intramolecularly cross-linked M13. The mass difference of ~634 u corresponds well with the mass increase caused by sulfo-SBED when both reactive sites have reacted with the peptide (Table 1). From the MALDI-TOF mass spectra recorded in linear mode it is evident that a significant amount of cross-linking product between CaM and M13 is created after UV irradiation times of at least 120 min, with the cross-linked CaM/M13 (1:1) complex containing between two to five cross-linkers. Moreover, CaM was found to be intramolecularly modified with one to five molecules of sulfo-SBED (Figure 2b).

Identification of Reaction Products

After the cross-linking reaction, reaction mixtures were subjected to enzymatic *in-solution* digestion with trypsin. These mixtures were rather complex as they contained peptides derived from CaM and M13 in addition to inter- and intramolecular cross-linking products as well as peptides modified by sulfo-SBED (Table 1). To enrich cross-linker containing species, peptide mixtures were subjected to affinity purification using monomeric avidin beads. In Figure 3, MALDI-TOF mass spectra from various stages of the avidin separation are presented, at the example of the reaction mixture of CaM and M13, which had been treated with 100-fold excess of sulfo-SBED and irradiated for 8 h. The spectrum in

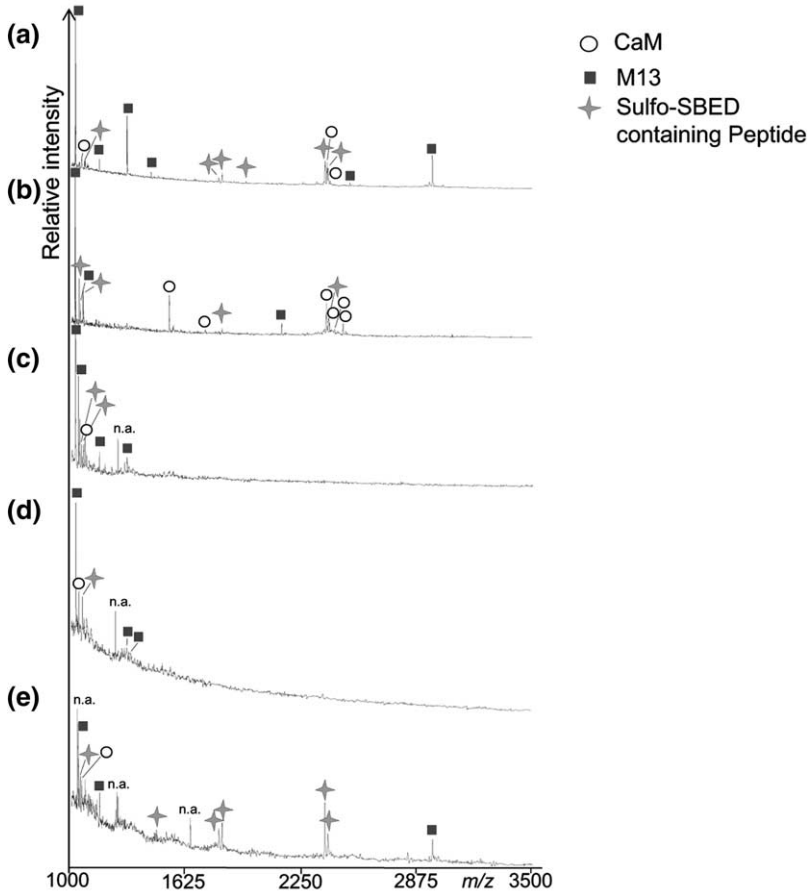


Figure 3. MALDI-TOF mass spectra of different fractions from avidin purification of CaM/M13 reaction mixture (100-fold excess sulfo-SBED, UV irradiation time 8 h). (**a**) Total cross-linking reaction mixture before avidin purification; (**b**) supernatant; (**c**) first phosphate fraction; (**d**) first fraction eluted with 15% methanol in 50 mM ammonium bicarbonate; (**e**) first fraction eluted with 50% aqueous acetonitrile in 0.4% formic acid. Signals of M13 (filled square) and CaM (open circle) peptides as well as cross-linker containing peptide species (filled diamond) are labeled; n.a.: signal not assigned.

Figure 3a presents the total reaction mixture before avidin separation, showing a number of peaks that were identified as tryptic peptides originating from both CaM and M13. Moreover, weak signals corresponding to sulfo-SBED modified peptides were observed in this spectrum. The MALDI-TOF mass spectrum (Figure 3b) of the supernatant exhibits similar signals, however, some signals of M13 peptides are missing, which might have been bound nonspecifically to the avidin beads. Three washes of the avidin beads with phosphate buffer yielded spectra as shown in Figure 3c for the first phosphate buffer fraction in which mainly unmodified peptide are detected. A subsequent wash with 15% methanol in 50 mM ammonium bicarbonate showed elution of a few CaM and M13 peptides (Figure 3d), but also some sulfo-SBED modified peptides were beginning to elute during the second washing step with methanol (data not shown). Finally, Figure 3e shows the MALDI-TOF mass spectrum of the solution (50% aqueous acetonitrile in 0.4% formic acid) that was used to elute biotinylated species from the beads. This spectrum predominantly contains sulfo-SBED derivatized CaM and M13 peptides, which had only been observed as weak signals in the original reaction mixture before avidin separation.

The fractions from the avidin separation were also analyzed by nano-HPLC/nano-ESI-FTICR mass spectrometry, as the FTICR technique offers unprecedented mass resolution and mass accuracy, thus allowing for a more confident assignment of cross-linking products. Figure 4 shows the deconvoluted ESI-FTICR mass spectrum of the avidin-purified peptide fraction (peptides eluted with 50% aqueous acetonitrile in 0.4% formic acid) of the above mentioned CaM/M13 reaction mixture. The cross-linker containing species are summarized in Table 2, confirming that more cross-linker-modified species are detected when UV irradiation times are increased from 60 min to 8 h. Strikingly, most of the assigned signals in the ESI-FTICR mass spectrum shown in Figure 4 originate from peptides that have been modified by sulfo-SBED. Merely one signal at *m/z* 2963.726 corresponds to intact, unmodified M13 peptide, which apparently had been retained on the avidin beads despite extensive washing steps described above.

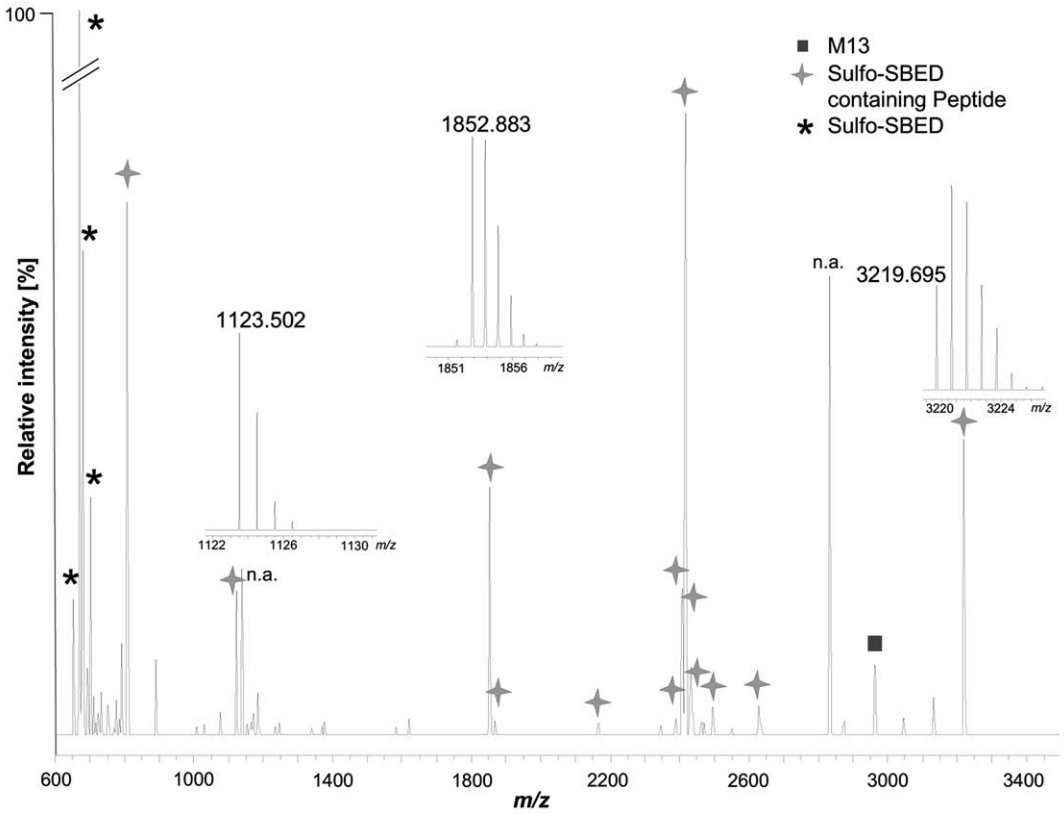


Figure 4. Deconvoluted ESI-FTICR mass spectrum of an avidin purified peptide mixture (first acetonitrile fraction, 100-fold excess of sulfo-SBED over protein/peptide concentration, irradiation time 8 h). Signals of cross-linker containing peptide species (filled diamond), reacted sulfo-SBED (filled star), and the M13 peptide (filled square) are labeled; n.a.: signal not assigned.

The high mass accuracy of the ICR technique presents an invaluable requirement for a correct assignment of cross-linker containing species. Based on MALDI-TOFMS data alone, the signal at *m/z* 2963.726 (Figure 4) might also have been assigned to a cross-linking product comprised of amino acids 4–19 of M13 and amino acids 75–77 of CaM. However, the calculated monoisotopic mass ($[M + H]^+$) of the respective cross-linking product is 2963.539 u, which deviates 63 ppm from the experimentally obtained mass. Another example for the necessity to rely on high mass accuracy data and on MS/MS data for a correct assignment of cross-linker-containing species presents the signal at *m/z* 2417.099, which could possibly correspond to an oxidization product of amino acids 107–126 of CaM (Table 1). However, the oxidation product of this peptide would have calculated monoisotopic mass ($[M + H]^+$) of 2417.174 u, which deviates by 37 ppm from the experimentally obtained value. Conclusively, this peptide can be confidently assigned to a sulfo-SBED modified peptide (amino acids 91–106), in which Lys-94 has reacted with the cross-linker. Alternatively, the signal at *m/z* 2417.099 could originate from a cross-linking product comprised of amino acids 1–2 of M13 and amino acids 75–86 of CaM. The unambiguous identification of the signal at *m/z* 2417.099 was achieved by tandem mass spectrometry showing b- and y-type ions of the CaM sequence 91–106 (VFDKDGNGYISAAELR) (Figure 5). The lysine residue at position 94 was identified as the amino acid that had reacted with SBED B-N_3 based on the mass shifts of the respective b- and y- type ions caused by reaction with the cross-linker (Δm 662.2 u). Unmodified y-type ions are observed from y_3 to y_{12}, whereas y_{13}, y_{14}, and y_{15} ions with the mass shifts caused by SBED B-N_3 are visible as doubly charged signals; b-type ions b_4 to b_{12} with the mass shift caused by SBED B-N_3 are observed as singly charged signals (Figure 5). This proves that lysine 94 is modified by the cross-linker. Lys-94 had already been found to be highly reactive in our previous studies on the CaM/M13 complex [10].

A signal at *m/z* 1084.490, appearing exclusively in the reaction mixture (100-fold excess of sulfo-SBED over protein/peptide concentration) that was irradiated for 120 min, was assigned to amino acids 17–19 of M13, which were modified by reaction of Lys-18 with the NHS ester group of sulfo-SBED (calc. $[M + H]^+$ at *m/z* 1084.489, Table 2). A cross-linker modified peptide consisting of amino acids 75–77 of CaM possesses a calculated monoisotopic mass ($[M + H]^+$) of 1084.456 u, which deviates by 31 ppm from the

Table 2. Summary of assigned signals in the deconvoluted ESI-FTICR mass spectra of avidin purified peptide mixtures (first acetonitrile fractions) using 100-fold excess of sulfo-SBED over protein/peptide concentration with irradiation times of 60 min, 120 min, and 8 h. The unambiguously assigned sulfo-SBED containing species are printed in shaded areas. The signal at *m/z* 2417.099 is marked with an asterisk, indicating that it was identified based on MS/MS experiments (Figure 5). Ox1, ox2: Oxidation products.

$[M+H]^+$ calc.	$[M+H]^+$ exp.	M13 Sequence	CaM Sequence	Type	Deviation [ppm]	Irradiation Time
809.326	809.326	1–1	–	Peptide + linker (SBED B-N_3)	0	8 hrs
	809.323				3	120 min
	809.324				2	60 min
965.427	965.427	1–2	–	Peptide + linker (SBED B-N_3)	0	120 min
1084.489	1084.490	17–19	–	Peptide + linker (SBED B-N_3)	−1	120 min
1123.500	1123.502	4–6	–	Peptide + linker (SBED B-N_3)	−2	8 hrs
	1123.502				−2	120 min
	1123.496				3	60 min
1842.881	1842.886	6–16	–	Peptide + linker (SBED A)	−3	120 min
1852.877	1852.883	6–16	–	Peptide + linker (SBED B-N_3)	−3	8 hrs
1868.872	1868.880	6–16	–	Peptide + linker (SBED B-N_3 ox1)	−4	8 hrs
2167.051	2167.059	4–16	–	Peptide + linker (SBED B-N_3)	−4	8 hrs
2389.073	2389.085	–	116–126 75–77	X-Link (SBED)	−5	8 hrs
2407.088	2407.097	–	91–106	Peptide + linker (SBED A or SBED B-NH_2 ox1)	−4	8 hrs
	2407.100				−5	120 min
2417.084	2417.099	–	91–106*	Peptide + linker (SBED B-N_3)	−7	8 hrs
	2417.098				−6	120 min
2417.108	2417.099	1–2	75–86	X-Link (SBED)	4	8 hrs
	2417.098				4	120 min
2433.078	2433.090	–	91–106	Peptide + Linker (SBED B-N_3 ox1)	−5	8 hrs
2433.103	2433.090	1–2	75–86	X-Link (SBED, ox1)	5	8 hrs
2439.078	2439.081	–	91–106	Peptide + linker (SBED A ox2)	−1	8 hrs
2497.108	2497.129	–	14–30	Peptide + linker (SBED A or SBED B-NH_2 ox1)	−8	8 hrs
2497.144	2497.129	1–5	75–77	X-Link + Linker (SBED A ox1)	6	8 hrs
2523.419	2523.421	4–26	–	Peptide	−1	120 min
	2523.404				6	60 min
2635.224	2635.210	17–19	1–13	X-Link (SBED ox1)	5	8 hrs
2635.234	2635.210	1–6	75–77	X-Link + Linker (SBED B-N_3 ox1)	9	8 hrs
2635.191	2635.210	–	75–77 1–13	X-Link (SBED ox2)	−7	8 hrs
2963.717	2963.726	1–26	–	Peptide	−3	8 hrs
	2963.713				1	120 min
	2963.701				5	60 min
3219.664	3219.695	3–19	87–90	X-Link (SBED)	−10	8 hrs
	3219.700				−11	120 min

measured mass and is therefore excluded as possible reaction product.

One intense signal at *m/z* 2832.518 (Figure 4) was not identified. A possible cross-linking product between amino acids 3–16 of M13 and amino acids 87–90 of CaM possesses a calculated monoisotopic mass ($[M + H]^+$) of 2832.401 u. However, as the calculated mass differs from the experimentally obtained mass by ca. 40 ppm, we were not able to unambiguously identify the ion underlying this signal. Each of the three signals at *m/z* 2433.090, 2497.129, and 2635.210 could not conclusively be assigned to a single cross-linker containing species; therefore, several possibilities are given in Table 2. It is certain, however, that the respective signals do not correspond to unmodified peptides, neither from CaM nor from M13.

One cross-linking product was identified between CaM (amino acids 87–90) and M13 (amino acids 3–19), pointing to lysine residues in positions 5, 6, or 18 of M13 that had most likely reacted with the alanine residue in position 88 of CaM. The distances between the N atoms of these lysine residues in M13 and the C-atom of Ala-88 in CaM are 13.6, 10.8, and 12.5 Å, respectively, according to the NMR structure of this complex [15]. The distance between the photo-reactive and the amino-reactive function within sulfo-SBED is 22.8 Å [23], and due to its inherent flexibility it seems to be able to cross-link reactive groups that are within shorter distances from each other.

It seems noteworthy that signals with high intensities at *m/z* 655.242 (calculated *m/z* 655.240) and *m/z* 671.236 (calculated *m/z* 671.235) were assigned to sulfo-

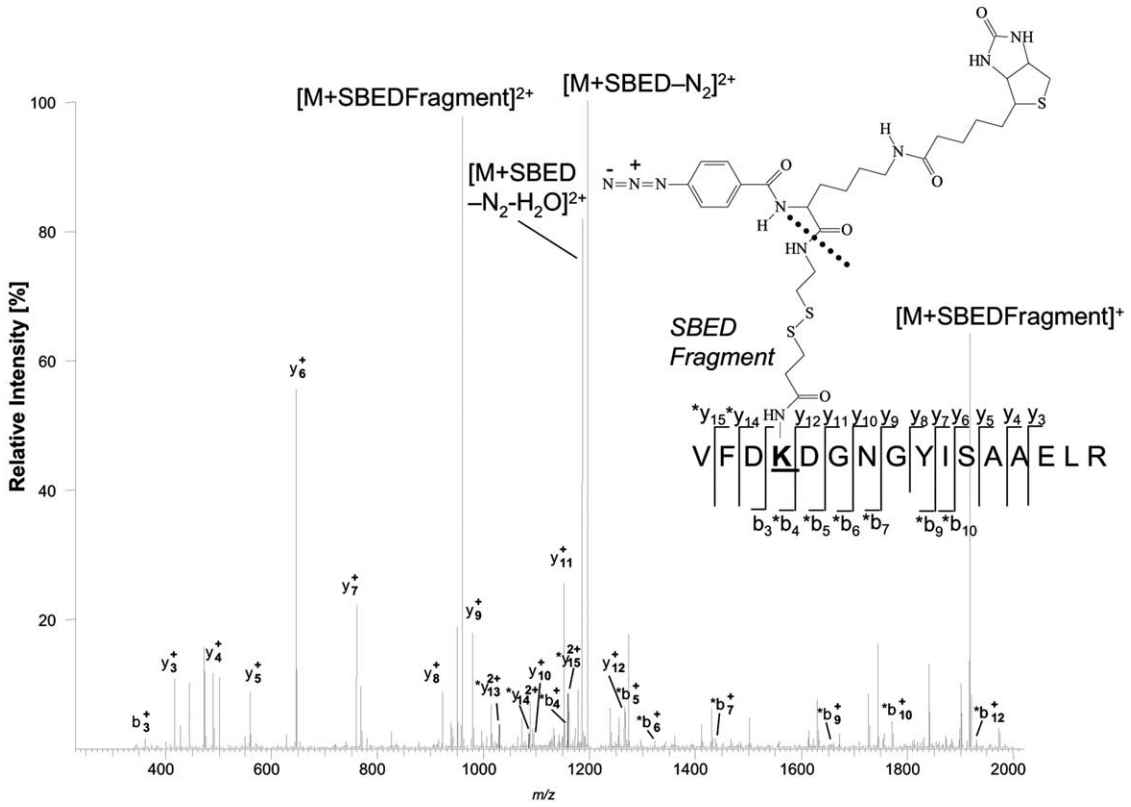


Figure 5. MS/MS of the signal at *m/z* 2417.099, which unambiguously identifies the peptide as CaM peptide comprising amino acids 91–106 with Lys-94 modified by SBED B-N_3 (see also Table 2). Fragment ions that show SBED B-N_3 modification are marked with an asterisk. Fragmentation of the cross-linker occurs preferably at the amide bond (indicated as "SBED Fragment"). N_2-loss from the azide group of sulfo-SBED is also observed.

SBED and its respective oxidation product in which the azide is converted to an amine group and the NHS ester moiety is hydrolyzed (Figure 4). Two other highly abundant signals at *m/z* 681.232 (calculated *m/z* 681.231) and *m/z* 697.224 (calculated *m/z* 697.225) were assigned to sulfo-SBED and its oxidation product in which the NHS group is hydrolyzed, whereas the azide group stayed intact. Although we attempted to remove excess sulfo-SBED after the cross-linking reaction with CaM, the presence of highly intense sulfo-SBED species and a number of modified M13 peptides in which the NHS ester function of sulfo-SBED had reacted (designated as SBED B-NH_2 and SBED B-N_3 species, Table 2), demonstrates that the procedure of removing excess cross-linker has to be improved. For future experiments, we are planning to employ size exclusion chromatography instead of filtration by Microcon filters for sulfo-SBED removal.

The results presented herein are encouraging and clearly demonstrate that our strategy to enrich cross-linker containing species by affinity purification is highly successful in identifying cross-linking products in an efficient and rapid way. For a confident assignment of cross-linker containing species, the application of high-resolution and high mass accuracy methods, such as FTICR mass spectrometry, is a valuable prerequisite. However, with the newest Q-TOF instruments that offer mass accuracies that begin to approach these of FTICR mass spectrometers, it might be feasible to analyze cross-linking products about as well, particularly in view of the MS/MS capabilities of Q-TOF mass spectrometers.

Conclusions

In this study, we successfully enriched cross-linker containing species by affinity purification after the cross-linking reaction. In combination with FTICR mass spectrometry, this strategy might be employed for a more efficient identification of interacting amino acid sequences in protein complexes. MS/MS experiments should be conducted to identify the exact points of cross-linking, which in future studies could be achieved by employing hybrid FTICR mass spectrometers offering the possibility to select and fragment ions before the ICR cell. Moreover, we are planning to employ cross-linkers that are both isotope-labeled and biotinylated, and which most likely will greatly facilitate an unambiguous identification of cross-linking products in case large protein assemblies are investigated.

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