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Peptide labeling with photoactivatable trifunctional cadaverine derivative and identification of interacting partners by biotin transfer

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ABSTRACT

A new photoactivatable trifunctional cross-linker, cBED (cadaverine-2-[6-(biotinamido)-2-(p-azidobenzamido) hexanoamido]ethyl-1,3′-dithiopropionate), was synthesized by chemical conversion of sulfo-SBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido) hexanoamido]ethyl-1,3′-dithiopropionate) with cadaverine. This cross-linker was purified by reversed-phase high-performance liquid chromatography (RP–HPLC) and characterized using matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) analysis. cBED is based on sulfo-SBED that has a photoactivatable azido group, a cleavable disulfide bond for label transfer methods, and a biotin moiety for highly sensitive biotin/avidin detection. By ultraviolet (UV) light, the azido group is converted to a reactive nitrene, transforming transient bindings of interacting structures to covalent bonds. In contrast to the sulfo-*N*-hydroxysuccinimide (sulfo-NHS) moiety of sulfo-SBED, which attaches quite unspecifically to amino groups, cBED includes a cadaverine moiety that can be attached by transglutaminase more specifically to certain glutamine residues. For instance, thymosin β_4 can be labeled with cBED using tissue transglutaminase. By high-resolution HPLC/ESI–MS (electrospray ionization–mass spectrometry) and tandem MS (MS/MS) of the trypsin digest, it was established that glutamine residues at positions 23 and 36 were labeled, whereas Q39 showed no reactivity. The covalent binding of cBED to thymosin β_4 did not influence its G-actin sequestering activity, and the complex could be used to identify new interaction partners. Therefore, cBED can be used to better understand the multifunctional role of thymosin β_4 as well as of other proteins and peptides.

Chemical cross-linking reactants are useful tools for analysis of protein interactions, and their field of application was increased by the development of photoactivatable heterobifunctional cross-linkers [1]. These types of cross-linkers possess a reactive group for a first conjugation to a protein, for example, sulfo-*N*-hydroxysuccinimide ester (sulfo-NHS ester).[1] The second functional group remains unreactive until photoactivation and is converted into highly reactive species that can react with structures or proteins in close vicinity. Such photoactivatable groups are aryl azides that are activated to nitrenes that can potentially react with several chemical moieties [2]. Nitrenes can insert in C—H or N—H and add to C=C bonds [3]. Rearrangement of the nitrene to the corresponding seven-membered, more stable ketenimine will take place after 10^{-4} s if no suitable reactive site is nearby [4]. This ketenimine is very reactive to nucleophiles [4]. Because of this broad reactivity, it is possible for aryl azides to react with a variety of biomolecules [5].

Cross-linkers that contain a reducible disulfide bond between the two functional groups can be used for label transfer experiments. Thereby, the part of the cross-linker containing the photoactivatable moiety is transferred to the targeted protein after reducing the disulfide bond. This strategy can be used to identify binding regions.

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[1] *Abbreviations used:* sulfo-NHS ester, sulfo-*N*-hydroxysuccinimide ester; sulfo-SBED, sulfosuccimimidyl[2-6-(biotinamido)-2-(*p*-azido-benzamido)-hexanoamido]-ethyl-1,3′-dithioproprionate; cBED, cadaverine-2-(6-[biotinamido]-2-(*p*-azido benzamido)-hexanoamido)-ethyl-1,3′-dithioproprionate; TFA, trifluoroacetic acid; StreptHRP, horseradish peroxidase-labeled streptavidin; RP–HPLC, reversed-phase high-performance liquid chromatography; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ESI, electrospray ionization; MS/MS, tandem MS; PBS, phosphate-buffered saline; BSA, bovine serum albumin; UV, ultraviolet; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline with Tween 20.

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Photoactivatable heterotrifunctional cross-linkers include an additional third functional group. This is, for instance, a coupled biotin moiety, which makes it possible to detect and separate cross-links via the highly sensitive biotin/avidin system.

Sulfo-SBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido) hexanoamido]ethyl-1,3′-dithiopropionate) is a photoactivatable heterotrifunctional cross-linker containing the photoactivatable aryl azide group, a biotin moiety, and a disulfide bond [6,7]. It is chemically cross-linked to proteins via a sulfo-NHS ester that reacts with primary amine groups to isopeptide bonds.

In this study, we describe the synthesis of a novel photoactivatable heterotrifunctional cross-linker, cBED (cadaverine), that combines the characteristics of sulfo-SBED and cadaverine. It has been shown that cadaverine serves as an amine substrate for transglutaminases [8,9]. These enzymes are Ca^{2+} dependent and catalyze an acyl transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and primary amine groups [10]. The specificity for the amine substrates is broad, whereas the number of proteins whose glutamine residues can form the acyl-enzyme intermediate is limited [10]. The bonds formed by transglutaminases are covalent, stable, and resistant to proteolysis.

The applicability of the novel cross-linker cBED was assessed on thymosin β_4, a small acidic peptide. This peptide was chosen because it is a known glutamine substrate of tissue transglutaminase [11]. Furthermore, thymosin β_4 sequesters monomeric actin (G-actin). The formation of a 1:1 complex along the subdomains 1 to 3 inhibits the polymerization to filamentous actin (F-actin) [12,13]. The effectiveness of cBED's photoactivatable group was tested using this interaction partner of thymosin β_4. Another advantage using thymosin β_4 is its presence in nearly every cell type with the exception of erythrocytes [14]. Therewith, the new cross-linker cBED could in the future be used to identify further interaction partners of thymosin β_4 as well as partners of other proteins.

Materials and methods

Proteins and reagents

Reagents were obtained from the following sources. Sulfo-SBED was obtained from Thermo Scientific Pierce (Schwerte, Germany). Thymosin β_4 was a gift from RegeneRx (Rockville, MD, USA). G-actin was prepared from bovine heart according to Pardee and Spudich [15]. LiChroprep RP-18 column (40–63 μm), acetonitrile, and trifluoroacetic acid (TFA, Uvasol) were obtained from Merck (Darmstadt, Germany). Guinea pig liver transglutaminase was obtained from Zedira (Darmstadt, Germany). Trypsin, cadaverine, and horseradish peroxidase-labeled streptavidin (StreptHRP) were obtained from Sigma–Aldrich (Schnelldorf, Germany). Fluorescamine was obtained from Serva (Heidelberg, Germany). Streptavidin magnetic beads were obtained from Hyglos (Bernried, Germany).

Preparation of cBED

Cadaverine dihydrochloride (40 mg, 228.4 μmol) was dissolved in 10 ml of borate buffer (50 mM borate/LiOH, pH 8.5). The reaction was started by the addition of 10 mg (11.4 μmol) sulfo-SBED in dimethylformamide. After incubation for 30 min at room temperature, the derivatized cadaverine was separated from excess cadaverine by solid-phase extraction on an RP-18 column. Unreacted cadaverine does not bind and is washed off by 0.1% (v/v) aqueous TFA, whereas the derivative is eluted by 0.1% (v/v) TFA in acetonitrile/water (60:40, v/v). Different cBED chromatographic purifications were combined and concentrated in vacuo. The concentration of cBED was determined photometrically at 269 nm, assuming an extinction coefficient of 19,400 L mol^{-1} cm^{-1}.

Enzymatic labeling of thymosin β_4 with cBED

cBED (575 μg, 750 nmol) and thymosin β_4 (250 μg, 50 nmol) were dried in vacuo and dissolved in 150 μl of reaction buffer (50 mM Tris–HCl [pH 7.4] and 15 mM $CaCl_2$), and the reaction was started by the addition of 2.5 U of guinea pig liver transglutaminase (in 100 μl of water). Reaction progress was monitored by analytical high-performance liquid chromatography (HPLC) after 0, 1, and 2 h. Thereby, 1 μl of the reaction mixture was diluted 1:1000 with 0.1% (v/v) TFA and analyzed using reversed-phase HPLC (RP–HPLC). The reaction was stopped after 2 h by adding 3 μl of 10% (v/v) TFA. The reaction products are referred to as $T\beta_4$–$cBED_1$ and $T\beta_4$–$cBED_2$.

RP–HPLC

All RP–HPLC experiments were carried out on a Merck Hitachi apparatus. A Beckman Ultrasphere ODS (5 μm, 4.6 × 250 mm) was used as the chromatographic column. The flow rate was 0.75 ml/min using the following eluents: 0.1% aqueous TFA (eluent A) and 0.1% TFA in acetonitrile/water (60:40, v/v) (eluent B).

For analytical HPLC, the applied gradient was linear from 10 to 100% B in 30 min. Detection was done by postcolumn derivatization with fluorescamine as described previously [16]. In the case of preparative RP–HPLC, the gradient was linear from 20 to100% B within 30 min. The fractions were collected every minute, and signals were detected using a diode array detector set at 220 and 270 nm.

Mass spectrometry and data analysis

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) was carried out as already described by Huff and coworkers [8]. Mass spectra were deconvoluted manually, and experimental mass values were compared with the calculated masses using ChemBioDraw Ultra 13.0 and PeptideMass programs (http://web.expasy.org/peptide_mass).

For tryptic digestion and high-resolution HPLC–electrospray ionization (ESI)–MS experiments, 2 μg of each protein, thymosin β_4 or $T\beta_4$–$cBED_1$, was dissolved in 10 μl of trypsin solution (20 μg/ml trypsin, 28 mM NH_4HCO_3, and 10% acetonitrile) and incubated for 20 h at 37 °C. This solution was directly analyzed by high-resolution HPLC–ESI–MS/MS (tandem MS) on an LTQ Orbitrap XL apparatus as described previously [13]. The eluents for reversed-phase chromatography were as follows: 0.1% aqueous formic acid (eluent A) and 0.1% formic acid in acetonitrile/water (80:20, v/v) (eluent B). The applied gradient was as follows: 0 to 1 min, 2 to 12% B; 1 to 16 min, 12 to 65% B (linear); 16 to 17 min, 65 to 100% B (linear). A flow rate of 80 μl/min was used.HPLC chromatograms of the tryptic in-gel digest of thymosin β_4 and $T\beta_4$–$cBED_1$ were compared and analyzed using MS Digest (ProteinProspector, version 5.10.2) for theoretical tryptic fragmentation. Putative cross-linking sites were analyzed in the MS/MS modus and compared with the theoretical fragmentation pattern using MS Tag (ProteinProspector, version 5.10.2).

Preparation of rat liver extract

Freshly prepared rat liver (3 g) was homogenized in 12 ml of ice-cold phosphate-buffered saline (PBS). The solution was divided into several cups and centrifuged (21,000*g*, 5 min, 4 °C). The supernatant is referred to as rat liver extract.

Cross-linking experiments

For the cross-linking experiments 120 ng of $T\beta_4$–$cBED_1$ (21 pmol) was dried in vacuo and different proteins were added.