Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Extending the cross-linking/mass spectrometry strategy: Facile incorporation of photo-activatable amino acids into the model protein calmodulin in *Escherichia coli* cells

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ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 6 February 2015
Accepted 22 February 2015
Available online xxxx

Keywords:
E. coli
Photo-amino acids
Photo-methionine
Photo-cross-linking
Mass spectrometry
Protein 3D-structure
Protein–protein interaction

ABSTRACT

Photo-induced cross-linking is a highly promising technique to investigate protein conformations and protein–protein interactions in their natural cellular environment. One strategy relies on the non-directed incorporation of diazirine-containing photo-activatable amino acids into proteins and a subsequent cross-link formation induced by UV-A irradiation. The advantage of this photo-cross-linking strategy is that it is not restricted to lysine residues and that hydrophobic regions in proteins can also be targeted, which is advantageous for investigating membrane proteins. Here, we present a simplified protocol that relies on the use of mineral salts medium without any special requirements for the incorporation of photo-methionines into proteins in *Escherichia coli* cells. The possibility to perform these experiments in *E. coli* is especially valuable as it is the major system for recombinant protein production. The method is exemplified for the Ca^{2+} regulating protein calmodulin containing nine methionines, which were found to be replaced by their photo-activatable analogues. Our protocol allows the facile and stochastic incorporation of photo-methionines as the basis for conducting photo-cross-linking experiments in *E. coli* in an efficient manner.

1. Introduction

The elucidation of protein–protein interactions in their natural environment presents one of the most important and pertinent tasks to allow a detailed understanding of the complex protein networks in a cell [1]. Protein–protein interactions in living cells are usually identified using affinity-based methods, such as co-immunoprecipitation or tandem-affinity purification (TAP) approaches [2]. As these methods rely on cell lysates as starting material, they are prone to the detection of false-positives resulting from a disruption of protein complexes during cell lysis. Also, transient binding partners might get lost during the washing procedures. As an alternative, chemical cross-linking methods have been developed to covalently fix protein complexes in living cells [3–6]. After the cross-linking reaction, the proteins of interest are usually enzymatically digested and the resulting peptide mixtures are analyzed by mass spectrometry (MS) in a "*bottom-up*" approach. The obtained cross-links allow determination of not only the identities of the binding partners, but also the position of the cross-link reveals the interaction site between two proteins.

In 2005, photo-activatable amino acid analogues with a diazirine group, termed photo-leucine, photo-isoleucine, and photo-methionine, were introduced [7]. They are particularly attractive as they allow cross-linking to proceed in time- and location-specific ways. Upon irradiation with UV-A light, a covalent linkage is created between those proteins that contain the photo-activatable amino acid. Incorporation of these diazirine-containing photo-activatable amino acid analogues into proteins can be achieved by adding them directly to the growth medium because their modified chemical structure does not interfere with them being in recognition by the cellular translation machinery. In the initial report, the photo-activatable amino acids were successfully incorporated into

Abbreviations: CaM, calmodulin; CID, collision-induced dissociation; DMEM-LM, Dulbecco's Modified Eagle's Limiting Medium; DTT, dithiothreitol; ESI, electrospray ionization; FA, formic acid; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, lysogeny broth; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; OD, optical density; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TAP, tandem-affinity purification; TFA, trifluoroacetic acid.

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http://dx.doi.org/10.1016/j.ymeth.2015.02.012
1046-2023/

proteins in HeLa cells demonstrating that they are non-toxic to the cells [7]. In the following years, the spectrum of cellular systems used was extended to human A549 cells [8], HEK 293 cells [9], and *Escherichia coli* cells [10,11]. The inherent benefit of a non-directed incorporation of photo-activatable amino acids is that in principle every methionine or leucine residue in a protein can be replaced by photo-methionine or photo-leucine. As diazirines react non-specifically, also hydrophobic protein regions are accessible for the photo-cross-linking reaction, which increases the amount of structural information that can be obtained from a cross-linking experiment compared to the use of amine-reactive cross-linkers [6]. The combined use of photo-activatable amino acid analogues with a mass spectrometric analysis of the generated photo-cross-linked products is a particularly effective experimental approach [9,12,13]. MS analysis of photo-cross-linked peptides is advantageous in many respects: First, the mass of the protein or the protein complex under investigation is theoretically unlimited as an enzymatic digestion is performed after the cross-linking reaction and MS analysis is performed at the peptide level. Second, MS analysis is rapid and requires only low amounts of protein, and finally, the protein's conformation and flexibility are accurately reflected as the cross-linking reaction is executed in the native or native-like environment. Not only can insights into the tertiary structures of proteins or on protein–protein interfaces be obtained [9], but the photochemistry of the diazirine also allows access to secondary structural information of the proteins under investigation [12,13].

When mapping the laminin γ1/nidogen-1 interaction using photo-activatable amino acids, we have been able to obtain incorporation rates of ca. 35% for photo-Met, but only ca. 3% for photo-Leu in HEK 293 cells [9,14]. Upon UV-A irradiation, the photo-cross-linking reaction was induced resulting in a covalent fixation of the protein–protein interaction. The laminin/nidogen complexes were enzymatically digested and the peptide mixtures generated were analyzed by high-resolution MS, resulting in the identification of several cross-linked sites. The distance constraints imposed by the photo-cross-links were combined with data obtained from complementary amine-reactive cross-linkers and served as a basis for deriving structural models of the laminin γ1/nidogen-1 complexes [9].

Photo-Met has also been successfully incorporated into proteins in *E. coli* cells [10,11]. The possibility to incorporate photo-activatable amino acids into proteins in *E. coli* is especially valuable as it is the main system for producing recombinant proteins. For this, DMEM-LM (Dulbecco's Modified Eagle's Limiting Medium), depleted of leucine and methionine, was employed. Incorporation rates were determined at different time points, showing incubation times between one to two hours to yield optimum incorporation rates. Combined with an MS analysis of the photo-cross-linked products, structural insights were obtained into 14-3-3ζ protein [10] and cytochrome P450 (CYP) 2B4/photo-cyt b5 complexes [11].

In this paper, we present a protocol that relies on the use of mineral salts medium instead of DMEM or dialyzed fetal bovine serum for the incorporation of photo-Met into proteins in *E. coli* cells. The advantage of our protocol is that cells are grown on mineral salts medium from the beginning, eliminating the complicated steps that are required in existing protocols (incubation of the cells in LB medium, followed by washing steps and further incubation in depleted medium). The feasibility of our simplified method is demonstrated for the Ca^{2+}-regulating protein calmodulin (CaM), which contains nine methionines [15]. We show that our protocol allows a facile incorporation of photo-amino acids, which is the basis for efficiently conducting photo-induced cross-linking/MS experiments and making it a valuable addition to the arsenal of cross-linking methods.

2. Materials and methods

2.1. Materials

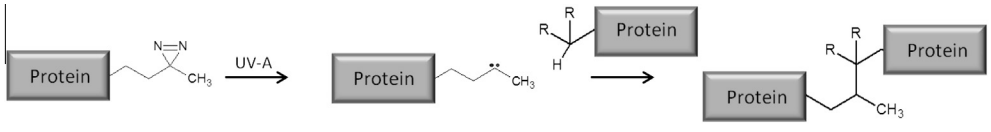
Photo-Met was purchased from Thermo Scientific. The proteases trypsin (porcine) and GluC were obtained from Promega. Chemicals were purchased from Sigma Aldrich and Merck. Solvents used for HPLC were LC-MS grade (VWR). MilliQ water was produced by a TKA Pacific system with X-CAD dispenser (Thermo Electron LED GmbH, part of Thermo Scientific).

2.2. Expression and purification of photo-Met-labeled CaM

Recombinant mammalian CaM with *C*-terminal His_6-tag (Supplementary Material, Scheme S1) was expressed in the *E. coli* strain BL21(DE3), using a pET28-a plasmid. The *E. coli* cells were grown in the dark (4.5 h) on mineral salts medium (1 L in a 3-L flask; shaking at 125 rpm; composition of medium is described in the Supplementary Material) up to an OD_{600nm} of 0.6 at 37 °C. Protein production was induced by adding IPTG to a final concentration of 0.1 mM. Simultaneously, photo-Met (30 mg/L) was added and the cells were further incubated at 37 °C in the dark. His_6-tagged CaM was enriched from cell extracts using immobilized metal ion affinity chromatography (HisTrapFF, 1 ml, GE Healthcare) and size exclusion chromatography (Superdex 75 pg 16/600, GE Healthcare) using an ÄKTA FPLC system (GE Healthcare). The amino acid sequence as well as incorporation of photo-Met into CaM was confirmed by peptide fragment fingerprint analysis. The final protein yield was ~3.8 mg/g wet weight.

2.3. Quantification of photo-Met incorporation

For every time-point after induction, 4.4 µg of CaM were used for MS analysis. In-solution digestion was performed with trypsin and GluC (protein enzyme ratio 30:1). Peptides were analyzed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) coupled to a nano-HPLC system (Ultimate 3000 RSLC, Thermo Fisher Scientific). For quantification of photo-Met incorporation, peak areas of the monoisotopic signals of corresponding Met or photo-Met containing CaM peptides were compared. Only those peptides that were found in all samples and possessed a charge of 2+ were considered for quantification. Incorporation rates were calculated for each peptide by dividing the sum of precursor peak areas of photo-Met species (Supplementary Material, Scheme S2) by the whole sum of all observed precursor peak areas for the different Met- and photo-Met-containing peptide species.



Scheme 1. Reaction mechanism of photo-Met. The diazirine group is activated by UV-A irradiation, resulting in a reactive carbene that can react with CH and NH groups in proteins.