Analytica Chimica Acta 676 (2010) 60–67

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Accessibility governs the relative reactivity of basic residues in formaldehyde-induced protein modifications

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ARTICLE INFO

Article history:
Received 29 May 2010
Received in revised form 13 July 2010
Accepted 27 July 2010
Available online 3 August 2010

Keywords:
Protein cross-linking
Formaldehyde
Chemical modification
Mass spectrometry
Peptide fragmentation
Residue accessibility
Reactivity

ABSTRACT

Cross-linking of proteins in a complex requires the chemical modification of the proteins in order to form a covalent link. This can be achieved *in vivo* using formaldehyde as it is small and rapidly permeates the cell membrane. Previous model studies of the speed and specificity of the first step of this reaction on peptides have suggested that residue accessibility and sequence micro-environment play a significant role in the production of the reactive intermediate necessary for cross-linking. This dependency was therefore further investigated on model proteins, which contain a more complex tertiary structure. Under mild reaction conditions, similar to those used for *in vivo* protein cross-linking, it was found that the vast majority of modification occurred on lysines, tertiary structure and solvent accessible surface area played a major role in regulating the extent of formaldehyde-induced modifications, and that the modifications on a folded protein did not significantly affect its tertiary structural stability.

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1. Introduction

Chemical cross-linking, in combination with mass spectrometric product analysis, is emerging as a powerful tool to study the interactions and geometries of protein complexes [1–5]. Commonly applied cross-linking strategies rely on well-defined conditions *in vitro* to react homo- or heterobifunctional cross-linkers of characteristic linker lengths with purified proteins or protein complexes. Enzymatic digestion of the cross-linked protein complexes and mass spectrometric analysis of the resultant peptides are then carried out to determine the interaction sites of the proteins. Distinguishing cross-linking products from the bulk of unchanged peptides has turned out to be challenging, thus several strategies to highlight the cross-linked dipeptides, such as incorporating stable isotopes [6–10] or reporter groups [11–13] into the cross-linking reagent have recently been introduced, in addition to the development of dedicated affinity enrichment strategies [14] or computational search tools [15–17].

A major limitation of the *in vitro* cross-linking strategy is the lack of cellular context of the interactions. Three systematic issues may affect these types of studies in particular: the absence of additional proteins that directly or indirectly interact with one of the complex components or compete for binding; changes in the tertiary structure of the proteins due to considerably lower concentrations of other species, i.e. the absence of molecular crowding; and altered protein diffusion rates in dilute solutions relative to those observed in high-density cellular environments. Due to the intricate complexity of the cell, attempts to recreate the cellular environment *in vitro* will, at best, be approximations of the natural physiochemical properties. Performing the cross-linking reaction inside a living cell is a promising way to eliminate these influences [18–20]. A number of groups have reported the feasibility of this general approach using formaldehyde as a cross-linking reagent [21–27]. Owing to its small size, it rapidly permeates membranes and quickly diffuses inside cells, thus is readily available for cross-linking of protein complexes without the need for prior cell lysis. Formaldehyde cross-linking in live cells has been utilized successfully to enrich for bait proteins and many cross-linked interaction partners that could then be identified by mass spectrometry [22–25,27], yet cross-linked dipeptides, indicative of the actual interaction site, have not yet been reported [28].

This lack of knowledge about the cross-linking sites on proteins upon *in vivo* formaldehyde treatment can be attributed to two related challenges: the dipeptides containing this information are

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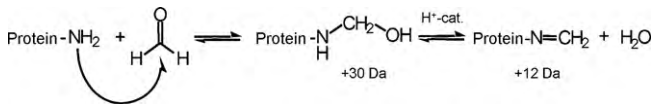
0003-2670/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.aca.2010.07.040



Scheme 1. The reaction mechanism for formaldehyde-induced modification of primary amino groups on proteins.

usually obscured by a complex matrix of more abundant non-cross-linked peptides, which is compounded by a limited understanding of what governs the reactivity of proteins under these conditions, and the resultant heterogeneity of the products. This work aims to add to the understanding of the reactions involved, by studying the products of the first step of the formaldehyde cross-linking process; Schiff-base formation (Scheme 1). Although two recent studies determined the products of the formaldehyde reaction with model peptides and proteins [29,30], the long formaldehyde exposure of several days used therein is not suitable for formaldehyde cross-linking inside cells, as it would lead to extensive protein modification, protein insolubility, and sample loss. We recently demonstrated that the side chains of lysine, tryptophan and cysteine, as well as amino-termini, were the dominant reactive sites in model peptides under conditions similar to an intracellular protein cross-linking reaction [31]. However, that study also indicated that accessibility and general micro-environment of reactive residues significantly influenced their reactivity.

The current study was designed to investigate whether complex tertiary structural arrangements, which are characteristic of proteins, influence the rate of the reaction between formaldehyde and modifiable residues (Scheme 1). The data suggest that modified proteins remain folded throughout the course of the reaction, accessibility has a large effect on the reaction rates for formaldehyde-induced modifications, and that tertiary structural stability is not significantly affected by the modification of amines to Schiff bases.

2. Materials and methods

2.1. Chemicals

Myoglobin from equine heart, carbonic anhydrase I (CA I) from human erythrocytes, lysozyme from chicken egg white, α-cyano-4-hydroxycinnamic acid (CHCA), dithiothreitol (DDT), trizma base, ammonium bicarbonate and sodium hydroxide were all purchased from Sigma (St. Louis, MO). Guanidine hydrochloride (Gdn HCl) was obtained from Schwarz/Mann Biotech (Cleveland, OH). Paraformaldehyde (PFA), formic acid (FA, 88%) and acetonitrile (ACN, HPLC grade) were purchased from Fisher (Fair Lawn, NJ). Endoproteinase Glu-C was obtained from Roche Applied Science (Penzberg, Germany). C4 and C18 extraction tips were obtained from Varian (Lake Forest, CA). 3 kDa MW-cut-off filters, 0.22 μm filters were purchased from Pall Corporation (Ann Arbor, MI). 3.5 kDa MW-cut-off dialysis cassettes were obtained from Pierce (Rockford, IL). Deionized water (18 MΩ cm) was prepared using a Nanopure Ultrapure Water System from Barnstead (Dubuque, IA).

2.2. Preparation of formaldehyde solution

A 4% (w/v) (1.3 M) formaldehyde stock solution was prepared by heating (80 °C) PFA in phosphobuffer saline (PBS, pH 7.5) for 30 min, cooling to room temperature and filtering through a 0.22 μm filter.

2.3. Reactivity of folded versus unfolded protein

CA I, lysozyme, and myoglobin (50–100 μM) were incubated and denatured with 6 M Gdn HCl in the presence of 0.33 M formaldehyde for 20 min. To reduce the disulfide bonds in lysozyme, 100 mM DTT was also added to the sample and heated for 1 h at 56 °C prior to reaction at room temperature with formaldehyde. Control samples were prepared by replacing the formaldehyde volume with PBS. Reactions were quenched by the addition of 1 M tris buffer (pH 7.5). Protein was isolated and concentrated using either C4/C18 extraction tips and eluted in 75:25 ACN:5% FA.

2.4. Change in reactivity with varying concentrations of Gdn HCl

Myoglobin (100 μM) was incubated as above with formaldehyde and 0 M, 0.5 M, 1 M, 2 M, 3 M, 4 M, 5 M, 6 M or 7 M Gdn HCl for 5 min. Control samples were prepared by replacing formaldehyde and Gdn HCl with PBS. Reactions were quenched as above. For these experiments a stock solution of Gdn HCl was prepared at 8 M in PBS and adjusted with sodium hydroxide to maintain physiological pH. For sample clean-up, see the appropriate section below.

2.5. Glu-C digestion

Proteins were digested overnight at room temperature in 50 mM ammonium bicarbonate (pH 8) with endoproteinase Glu-C. The digestion was quenched using 5% formic acid in water, lyophilized and resuspended in 0.01% formic acid in water.

2.6. Comparison of the electrospray charge state profile of folded untreated and folded, formaldehyde-treated myoglobin

Myoglobin (100 μM) was incubated in PBS (37 °C) in the presence or absence of 0.33 M formaldehyde for 50 min. The reactions were quenched as above. Proteins were washed and resuspended from the filter membranes using either water (pH 6) or 0.01% formic acid in water (pH 3). The protein resuspended in water (pH 6) was used to prepare the pH 4 and 5 samples by diluting with 0.001 and 1.75×10^{-4}% formic acid in water, respectively. All samples were diluted with ACN for analysis by ESI-MS (2000 Q Trap, Applied Biosystems, Foster City, CA). The average charge state for each spectrum was calculated using weighted peak areas for all charge states with signal to noise ratio greater than 5.

2.7. Unfolding myoglobin during reaction with formaldehyde

Myoglobin (100 μM) was incubated as above for 30 min at which time the protein was unfolded by addition of 3 M Gdn HCl. The reaction was continued for another 20 min. Aliquots were taken throughout the experiment at times between 5 and 50 min. Two similar experiments were performed, one without the addition of 3 M Gdn HCl and one with the addition of Gdn HCl at $t = 0$ min. All three experiments were compared to a control which lacked both Gdn HCl and treatment with formaldehyde. The reactions were quenched by the addition of 1 M tris buffer (pH 7.5).

2.8. Myoglobin sample clean-up

To avoid precipitation of the Gdn HCl-containing protein samples, they were subjected to dialysis prior to filtration. The Gdn HCl-containing samples were dialyzed (3.5 kDa cassettes) against 50 mM tris buffer for 2 h at room temperature. The dialysis buffer was changed and dialysis continued for another 2 h at room temperature. The dialysis buffer was changed again and the sample was dialyzed overnight at 4 °C. For the dialyzed samples and all other non-Gdn HCl-containing samples, myoglobin was isolated and concentrated using 3.5 kDa MW-cut-off filters. Proteins were washed and resuspended from the filter membranes using 0.01% formic acid in water.

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