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Ovalbumin labeling with *p*-hydroxymercurybenzoate: The effect of different denaturing agents and the kinetics of reaction

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ABSTRACT

The aim of our study was to investigate how denaturing agents commonly used in protein analysis influence the labeling between a reactive molecule and proteins. For this reason, we investigated the labeling of ovalbumin (OVA) as a globular model protein with *p*-hydroxymercurybenzoate (*p*HMB) in its native state (phosphate buffer solution) and in different denaturing conditions (8 mol L^{-1} urea, 3 mol L^{-1} guanidinium thiocyanate, 6 mol L^{-1} guanidinium chloride, 0.2% sodium dodecyl sulfate, and 20% methanol). In addition to chemical denaturation, thermal denaturation was also tested. The protein was pre-column simultaneously denatured and derivatized, and the *p*HMB-labeled denatured OVA complexes were analyzed by size exclusion chromatography (SEC) coupled online with chemical vapor generation–atomic fluorescence spectrometry (CVG–AFS). The number of –SH groups titrated greatly depends on the protein structure in solution. Indeed, we found that, depending on the adopted denaturing conditions, OVA gave different aggregate species that influence the complexation process. The results were compared with those obtained by a common alternative procedure for the titration of –SH groups that employs monobromobimane (mBBr) as tagging molecule and molecular fluorescence spectroscopy as detection technique.

We also investigated the labeling kinetics for denatured OVA and *p*HMB, finding that the 4 thiolic groups of OVA have a very different reactivity toward mercury labeling, in agreement with previous studies.

Thiolic (–SH)[1] groups in cysteine residues play major functional and structural roles in proteins, including disulfide bond formation, antioxidant activity, and metal ion binding, in the active group of enzymes but also with toxic heavy metals [1]. In this regard, the determination of thiolic groups in biological matrices became a significant analytical task for clinical, pharmacological, and environmental concerns [2]. The development of effective methods for –SH group quantitation in proteins has proven to be difficult because no specific physicochemical properties (e.g., strong ultraviolet–visible [UV–Vis] absorptions or native fluorescence) can be exploited for this purpose [2]. Therefore, methods based on the selective reaction of –SH groups with labeling agents have been proposed, with the most effective ones involving complexation with metal derivatives [3]. Due to the soft–soft interaction between mercury and sulfur, organomercury compounds have been recognized as very selective labeling reagents for thiolic group derivatization [4]. The reaction $RS^- + R'–Hg^+ \rightarrow RS–Hg–R'$ is exothermic and thermodynamically favorable, with a very large stability constant [5]. Hence, the mercury–protein complexes obtained are stable toward dissociation during chromatographic separation, thereby making possible their chemical characterization in either the native or denatured state of the protein [3,6]. Among the organic species of mercury, *p*-hydroxymercurybenzoate (*p*HMB) is a monofunctional, water-soluble, and nonvolatile reagent. Compared with inorganic mercury, *p*HMB has the advantage to form 1:1 complexes with thiols, whereas inorganic mercury is a potentially bifunctional reagent and usually yields mixtures of complexes (1:1 and 1:2 stoichiometry) [7].

After the derivatization with mercurial probes, thiol detection may occur with a chemical vapor generation (CVG) system coupled to atomic fluorescence spectrometry (AFS), one of the most

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[1] Abbreviations used: –SH, thiolic; UV–Vis, ultraviolet–visible; *p*HMB, *p*-hydroxymercurybenzoate; CVG, chemical vapor generation; AFS, atomic fluorescence spectrometry; LOD, limit of detection; OVA, ovalbumin; SEC, size exclusion chromatography; mBBr, monobromobimane; PBS, phosphate buffer solution; SDS, sodium dodecyl sulfate; GndSCN, guanidinium thiocyanate; GndHCl, guanidinium chloride; MeOH, methanol; MW, molecular weight; AF, atomic fluorescence; SS, disulfide.

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sensitive, selective, and low-cost techniques for mercury determination (limits of detection [LODs] ⩾ 0.1 ng/L) [8]. In the case of organic mercurial probes such as *p*HMB, the conversion of organomercury compounds (R–Hg–SR′) to inorganic Hg(II) prior to CVG–AFS is mandatory to obtain high sensitivity and reproducible results. Recently, we proposed a photochemical method for the online digestion of *p*HMB and its complexes with low-molecular-weight thiols [9] and proteins [10], followed by CVG–AFS detection.

The reactivity of thiolic groups in proteins plays a key role in labeling strategies and is strongly related to the structure of the protein in solution. Native proteins may have unreactive –SH groups buried in their structure and, hence, may be not accessible to the solvent medium. Denaturation completely alters the protein structure; as a result, several thiolic groups may become reactive and others may become not accessible [11]. Each denaturation protocol leads to different unfolded states of different compactness and, therefore, to a different availability of thiolic groups [12]. Thus, from the analytical point of view, denaturation may change the labeling yield and, therefore, the detection sensitivity. In addition, the use of denaturing agents in the sample preparation step of several bioanalytical procedures is important to improve protein solubility and the analyte recovery in raw matrices [13]. Thus, the thiol labeling of proteins in different denaturing conditions needs to be carefully investigated.

Chemical reagents for protein denaturation in labeling procedures belong to three classes: (i) salting-in (or chaotropic) agents, (ii) detergents, and (iii) organic solvents [13]. Acid denaturation is usually avoided both because it may lead to protein precipitates and because a low pH disadvantages –SH complexation. A basic medium, as reported in our recently published work [14], acts as denaturing media and simultaneously causes the hydrolysis of disulfide bonds. These parallel effects make difficult studying the pure denaturing action of the media unless the protein has no disulfide bond in its native state. Proteins can also be denatured by heat, which disturbs hydrogen bonds and nonpolar hydrophobic interactions.

Ovalbumin (OVA) is a 45-kDa protein characterized by a compact, globular structure. According to Brash and Horbett [15], OVA is classified as a "hard" protein and is not easily denatured. Thus, OVA represents a good model to evidence the differences in labeling when performed in different denaturing conditions with respect to "soft," easily denatured proteins. In addition, OVA is a good model for fundamental studies of protein labeling, having 4 nonoxidized thiolic groups theoretically available for complexation with *p*HMB (Cys34, −53, −373, and −388) [16]. Several studies on the titration of thiolic groups of native OVA with mercurial probes have been carried out, reporting a number of –SH groups titrated between 1 and 3.7 [3,17–21]. However, to the best of our knowledge, the conjugation of mercurial labeling techniques with the use of denaturants has not yet been applied to the study of OVA, and in general information on the labeling of protein thiols in different denaturing conditions is lacking.

In this work, we investigated the labeling process of OVA with *p*HMB in five different denaturing media and in the native state by coupling size exclusion chromatography (SEC) to CVG–AFS.

The results are compared with those obtained with monobromobimane (mBBr), an alternative widespread agent for the labeling of thiolic compounds. Thiols react with mBBr by nucleophilic substitution, yielding a highly fluorescent thioether [22]. We analyzed the OVA–mBBr complexes by SEC coupled to molecular fluorescence spectroscopy.

Then, we selected the denaturing conditions that guaranteed the best complexation yield, and we studied the kinetics of OVA–*p*HMB complexation. The results are compared with those previously published and are discussed in terms of analytical advantages/drawbacks of the use of denaturing agents and strategies in protein analysis.

Materials and methods

Chemicals

Analytical reagent-grade chemicals were purchased from Sigma–Aldrich (Milan, Italy), except where otherwise noted, and used without further purification. Ultrapure water was prepared with an ELGA PURELAB UV system (Veolia Environnement, Paris, France). For chromatographic analyses, all of the solutions were diluted to the appropriate concentration range immediately before injection.

A 1×10^{-2} mol L^{-1} stock solution of *p*HMB (CAS no. 138-85-2, $HOHgC_6H_4CO_2Na$) was prepared in 0.01 mol L^{-1} NaOH (TraceSELECT grade) to improve its solubility and was stored at 4 °C. Stock 60-μmol L^{-1} solution of mBBr (Sigma–Aldrich) was prepared in acetonitrile in an amber glass vial and stored at −20 °C. mBBr is photodegradable and gives fluorescent products that may interfere with thiol determination. Thus, the reactions need to be carried out in the dark.

Phosphate buffer solutions (PBSs) were prepared from $NaH_2PO_4 \cdot H_2O$ and K_2HPO_4 (BDH Laboratory Supplies, Poole, UK). The following denaturant solutions were prepared by dissolving the corresponding solid compound in water: 0.2% sodium dodecyl sulfate (SDS), 3 mol L^{-1} guanidinium thiocyanate (GndSCN), 6 mol L^{-1} guanidinium chloride (GndHCl), 8 mol L^{-1} urea, and 20% methanol (MeOH) (Carlo Erba, Rodano, Milan, Italy). Urea, GndSCN, and GndHCl solutions were stored at 4 °C; SDS solutions were kept at room temperature to avoid salt precipitation.

For SEC calibration, stock solutions of OVA (chicken egg, grade VI, purity ⩾98%, EC 232-692-7), thyroglobulin (EC 232-721-3), aldolase from rabbit skeletal muscle (EC 41-21-3), bovine serum albumin (A8531 EC 232-936-2), aprotinin (EC 232-994-9), and myoglobin (EC 309-705-0) were prepared in 0.1 mol L^{-1} PBS (pH 7.4). The precise protein concentrations were determined spectrophotometrically. For the labeling procedure, 5 μmol L^{-1} OVA stock solutions were prepared by dissolving lyophilized OVA in each denaturant solution.

Stock 2.5-mol L^{-1} solutions of $NaBH_4$ (pellets, reagent for AAS, minimum assay >96%, Merck, Whitehouse Station, NJ, USA) in 0.3% (m/v) NaOH were prepared, microfiltered (0.45-μm membrane), and stored at 4 °C. Diluted $NaBH_4$ solutions (0.05 mol L^{-1}) for the CVG system were prepared daily from the stock solutions. HCl solutions were prepared from 37 wt% HCl (Carlo Erba).

Safety considerations

*p*HMB is toxic. Inhalation and contact with skin and eyes should be avoided. All work should be performed in a well-ventilated fume hood.

Instrumentation and chromatographic conditions

SEC hyphenated with UV detection, in series with a combined molecular weight (MW)/UV reactor and a CVG–AFS system, was used for all of the measurements. This detection system provides a multidimensional analysis (UV–Vis absorbance and mercury-specific chromatograms and molecular weight distribution) from a single injection. The choice of SEC to study the denaturation of protein–*p*HMB complexes was determined by the fact that some of the new stationary phases in SEC have been proven to maintain the structural integrity of the coordination complexes in comparison with those employed in ion exchange chromatography and reversed phase chromatography.

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