Analytical Biochemistry 491 (2015) 43–51

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Evaluation of colorimetric assays for analyzing reductively methylated proteins: Biases and mechanistic insights

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ARTICLE INFO

Article history:
Received 16 July 2015
Accepted 25 August 2015
Available online 3 September 2015

Keywords:
Protein
Quantitation
Bradford assay
Bicinchoninic acid assay
Methylation
Lysine

ABSTRACT

Colorimetric protein assays, such as the Coomassie blue G-250 dye-binding (Bradford) and bicinchoninic acid (BCA) assays, are commonly used to quantify protein concentration. The accuracy of these assays depends on the amino acid composition. Because of the extensive use of reductive methylation in the study of proteins and the importance of biological methylation, it is necessary to evaluate the impact of lysyl methylation on the Bradford and BCA assays. Unmodified and reductively methylated proteins were analyzed using the absorbance at 280 nm to standardize the concentrations. Using model compounds, we demonstrate that the dimethylation of lysyl ε-amines does not affect the proteins' molar extinction coefficients at 280 nm. For the Bradford assay, the responses (absorbance per unit concentration) of the unmodified and reductively methylated proteins were similar, with a slight decrease in the response upon methylation. For the BCA assay, the responses of the reductively methylated proteins were consistently higher, overestimating the concentrations of the methylated proteins. The enhanced color formation in the BCA assay may be due to the lower acid dissociation constants of the lysyl ε-dimethylamines compared with the unmodified ε-amine, favoring Cu(II) binding in biuret-like complexes. The implications for the analysis of biologically methylated samples are discussed.

The Coomassie blue G-250 dye-binding (Bradford) and bicinchoninic acid (BCA) assays are common colorimetric assays used to determine the concentrations of proteins [1–3]. Both assays produce a colored solution in the visible spectrum in response to protein. The color formation observed in the Bradford assay is a result of interactions between the protein and the Coomassie blue G-250 dye (Fig. 1A). Under acidic conditions, the dye is red in its protonated state. Through electrostatic and hydrophobic interactions with a protein molecule, the anionic (−1 net charge) blue form of the dye is stabilized [4–7]. In the BCA assay, color formation is the result of two reactions. First, cupric ions (Cu^{2+}) are reduced to cuprous ions (Cu^{+}) by the protein under alkaline conditions via the biuret reaction [8]. Then, a complex between Cu^{+} and two molecules of bicinchoninic acid forms, giving the characteristic purple color (Fig. 1B) [2]. The intensity of the color formed by these assays is measured by absorbance photometry at 595 and 562 nm for the Bradford and BCA assays, respectively. Typically, standard solutions of bovine serum albumin (BSA) are used to produce a calibration curve of absorbance versus mass concentration. Assuming that the analyte proteins react in the same manner as the BSA standard, the unknown concentration can be determined. Unfortunately, the assumption that the assay sensitivity or response (absorbance per unit concentration) is universal is not always valid, and protein-to-protein variability can lead to an over- or underestimation of the analyte protein's concentration [9].

Protein composition can bias the results of Bradford and BCA assays. In the Bradford assay, the dye–protein interaction is influenced by the electrostatic interactions of the sulfonate groups with the basic residues, arginine and lysine [4,5]. An additional factor is the hydrophobic interactions of the dye with tryptophan, phenylalanine, and tyrosine residues [4,10]. Proteins that are largely hydrophobic and/or have a high proportion of arginine and lysine residues give higher absorbance values than the same mass concentration of a protein with less hydrophobic character and/or fewer basic residues. Similarly, the BCA assay can be influenced by

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; PTM, posttranslational modification; DMAB, dimethylamine–borane complex; NMR, nuclear magnetic resonance; HEWL, hen egg white lysozyme; DSS, sodium 3-(trimethylsilyl)-1-propanesulfonic acid; UV, ultraviolet; D_2O, deuterated water; DM-Lys, N^{ε},N^{ε}-dimethyl-L-lysine; PBS, phosphate-buffered saline; MALDI MS, matrix-assisted laser desorption/ionization mass spectrometry.

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http://dx.doi.org/10.1016/j.ab.2015.08.027
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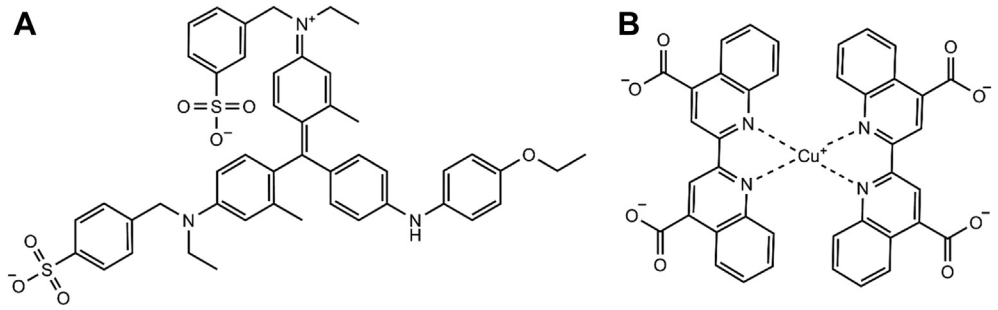

Fig.1. Structures of the Coomassie blue G-250 dye in its blue anionic (−1 net charge) form (A) and the purple (bicinchoninic acid)$_2$–Cu^+ complex (B).

the amino acid composition of the analyte protein. Cysteine, cystine, tyrosine, and tryptophan residues have been shown to participate in the reduction of Cu^{2+} to Cu^+ ions [11]. Proteins that contain a high proportion of these reactive residues will produce higher responses. Differences in the composition of the proteins and the standard, typically BSA, can result in inaccurate concentration determinations.

The influence of amino acid composition on protein concentration assays also includes effects from posttranslational modifications (PTMs). PTMs alter the chemical composition of a protein with the addition or cleavage of a functional group and are important for protein regulation and function. Glycosylation is a PTM where a mono- or polysaccharide is added to a protein, typically at asparagine, serine, or threonine residues. Fountoulakis and coworkers investigated the variability of the Bradford, BCA, and Lowry assays in quantifying several glycosylated proteins in comparison with the unmodified protein [10]. They concluded that glycosylation interfered with the assay responses, resulting in an underestimation of the glycoprotein concentration with the Bradford assay and an overestimation of the glycoprotein concentration with the BCA and Lowry assays [10].

Lysine methylation is a common PTM and can be mimicked using the reductive methylation reaction [12]. Lysine methylation is important for protein regulation and signaling [13] and can exist as mono-, di-, or trimethylation of the ε-amino group. Mono- and dimethylation of lysine residues can be produced chemically using the reductive methylation reaction. Reductive methylation selectively incorporates methyl groups at the lysyl ε-amino groups and the N-terminal α-amino group without disturbing the structure, and often activity, of the protein [12,14]. Formaldehyde reacts with the primary amines to form Schiff bases, which are subsequently reduced to form methyl groups with a reducing reagent such as dimethylamine–borane complex (DMAB) [15]. In the presence of excess reagents, the reaction adds two methyl groups to each primary amine. Reductive methylation is commonly used in X-ray crystallography because the modification rarely alters the protein's overall structure and aids crystallization [14]. The modification is also useful for studying proteins by nuclear magnetic resonance (NMR) spectroscopy because ^{13}C formaldehyde can be used to add ^{13}C methyl groups as isotopic labels [16,17]. The ^{13}C methyl groups are sensitive probes for investigating structural and dynamic properties of proteins and their complexes by NMR [16,18–39]. Because of the extensive use of reductive methylation in the study of proteins and the natural occurrence of methylated proteins, it is necessary to evaluate the impact of methylation on the response of commonly used concentration assays. In this study, we investigated the effect of lysine methylation on the determination of protein concentration by the Bradford and BCA assays. Amino acid composition and hydrophobicity can bias the responses of the Bradford and BCA assays, so reductive methylation may likewise alter the accuracy of these assays. Unmodified and reductively methylated hen egg white lysozyme (HEWL), BSA, and ovalbumin were used to test and compare the assays' responses.

Materials and methods

Materials

Acetonitrile, ammonium sulfate, BSA (cat. no. A7030), DMAB, formaldehyde, HEWL (cat. no. l6876), albumin from chicken egg white (ovalbumin, cat. no. A5503, grade V), potassium phosphate (monobasic and dibasic), sinapinic acid, trifluoroacetic acid, L-lysine hydrochloride, N^{ε},N^{ε}-dimethyl-L-lysine hydrochloride, anhydrous caffeine, and sodium 3-(trimethylsilyl)-1-propanesulfonic acid (DSS) were purchased from Sigma–Aldrich. The BCA assay kit and Coomassie Plus (Bradford) assay reagent were purchased from Fischer Scientific/Pierce. Corning ultraviolet (UV)-transparent half-area 96-well plates were purchased from VWR. Deuterated water (D_2O) was purchased from Cambridge Isotope Laboratories. Bio-Gel P-4 size exclusion chromatography resin was purchased from Bio-Rad. All water used was supplied from a Millipore Direct-Q 3 ultrapure water system.

Sample pathlength determination

Because a microplate spectrophotometer was used to measure the absorbance, the pathlength varied with the sample volume. The method of McGown and coworkers was used to determine the sample pathlength and is an option in the Gen5 software for the BioTek PowerWave XS2 microplate spectrophotometer used in this study [40]. Briefly, the absorbances of water at 977 and 900 nm are subtracted and divided by the known absorbance of water at 977 nm and 1 cm (0.180) to give the pathlength in centimeters (cm). The pathlengths of replicate samples of water at 100, 120, and 200 μl were measured in a UV-transparent half-area 96-well plate at 25 or 37 °C. The average pathlength values and 95% confidence intervals are summarized in Table S1 of the online supplementary material. The average pathlength values were used to normalize the measured absorbances to 1 cm.

Extinction coefficients at 280 nm

To determine whether lysine methylation added significantly to the overall absorbance at 280 nm (A_{280nm}) of a protein, the molar extinction coefficients at 280 nm (ε_{280} values) of L-lysine (Lys) and N^{ε},N^{ε}-dimethyl-L-lysine (DM-Lys) were determined in phosphate-buffered saline (PBS) buffer (50 mM potassium phosphate and 100 mM NaCl, pH 7.5). Solutions (50 g/L) of Lys and DM-Lys were prepared by weighing at least 10.0 mg of material and dissolving in