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Evaluation of employing poly-lysine tags versus poly-histidine tags for purification and characterization of recombinant copper-binding proteins☆

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ABSTRACT

Quantitative characterization of metalloproteins at molecular and atomic levels generally requires tens of milligrams of highly purified samples, a situation frequently challenged by problems in generating unmodified native forms. A variety of affinity tags, such as the popular poly-histidine tag, have been developed to facilitate purification but they generally rely on expensive affinity resins and their presence may interfere with protein characterization. This paper documents that addition of a poly-lysine tag to the C-terminus enables, for the copper-binding proteins examined, ready purification in large scale via cost-effective cation-exchange chromatography. The tag may be removed readily by the enzyme carboxypeptidase B to generate the native protein with no extra residues. However, this cleavage step is normally not necessary since the poly-lysine tag is shown to have no detectable affinity for either Cu(I) or Cu(II) and imposes no interference to the copper binding properties of the target proteins. In contrast, the poly-histidine tag possesses a sub-picomolar affinity for Cu(I) and -nanomolar affinity for Cu(II) and may need to be removed for reliable characterization of the target proteins. These conclusions may be extended to the study of other metallo-proteins and metallo-enzymes.

1. Introduction

More than 25% of all proteins require a transition metal ion for their biological function [1]. Quantitative evaluation of metal–protein interactions at an atomic level is the key to a molecular understanding of the role of the metal ion and the evaluation relies upon the availability of an adequate quantity of highly purified protein. This requirement can be compromised by the challenge of isolating the target protein in unmodified native form.

A variety of affinity tags have been developed to assist in detection and purification of recombinant proteins. These include epitopes *c*-myc, HA (hemaglutinin antigen), FLAG, strep-tags and poly-histidine tags [2,3]. Purification generally requires an expensive affinity resin. The tag may be removed post-purification and this is often necessary for proper characterization of the target protein. Hence, a specific protease site such as factor Xa, thrombin, TEV or HRV 3C is incorporated in the sequence between the affinity tag and the target protein. For example, the poly-histidine tag has been employed extensively in isolation of many copper-transporting proteins including CusA and CusB from *Escherichia coli* [4,5], the P-type ATPase CopA from *Legionella pneumophila* [6] and the related CopA and its partner chaperone CopZ from *Archaeoglobus fulgidus* [7–9]. However, in each of these examples, the poly-histidine tag was not removed (or could not be removed due to protection by detergents or lipids) from the purified proteins for structural and functional assays. The tag is expected to bind either Cu(I) or Cu(II) with considerable affinity and may interfere with the proper characterization of the copper binding and transporting properties of the target protein. None of these aspects has been evaluated and quantified.

Tagging of protein C-termini with poly-arginine or poly-lysine tags raises the isoelectric point but was reported to facilitate purification via conventional and cost-effective cation-exchange chromatography [10–16]. A comparative study of the purification of the same protein via poly-arginine and poly-histidine tags led to the conclusion that the former tag was superior [17]. A bonus of employing the poly-arginine or poly-lysine tags is that they can be removed completely from the C-terminus via the enzyme carboxypeptidase B to generate the unmodified protein form [18]. Proteolytic cleavage of other affinity tags usually leaves several linker residues behind. In addition, the length of the arginine tags or the lysine tags can be as short as a few residues only and so they may have little impact on protein properties and may not need to be removed. In fact, a deca-lysine tag was tagged to the C-terminus of the enzyme cyclodextrin glycosyltransferase from *Bacillus macerans* and was shown to have little effect on enzyme characteristics [13]. Some enzymes were reported to exhibit even better stability and catalytic efficiency upon immobilization onto negatively charged matrices via C-terminal ploy-lysine tags [14,16]. However, application of

☆ This paper is dedicated to the memory of our friend and colleague Professor Graeme Hanson.

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poly-arginine or poly-lysine tags on purification and characterization of metalloproteins and metalloenzymes has yet to be demonstrated.

Our group has expressed and characterized many soluble metal-binding proteins, especially those that bind copper [19]. Some of these share similar chromatographic properties with many *E. coli* host proteins and require multiple, tedious chromatographic procedures for their purification. In the current work, three representative copper-binding proteins were selected for a systematic evaluation of the use of poly-lysine tags versus poly-histidine tags for purification and characterization of recombinant forms. The selection included two protein variants of CopC from *Pseudomonas fluorescens* SBW25, namely, CopC-H85F and CopC-H1,85F [20]. They each feature a single Cu(II) binding site of sub-picomolar affinity. The third protein is N-terminal metal-binding domain 6 of human Wilson protein (WLN6) which possesses a Cu(I) binding site of sub-femtomolar affinity [21]. The study provided a useful guideline in applying these affinity tags to purification and characterization of copper-binding proteins and the conclusions are likely applicable to other metalloprotein systems as well.

2. Experimental section

2.1. Materials and general methods

General chemicals and reagents including Cu(I) ligands ferrozine (Fz), bicinchoninic anion (Bca) and bathocuproïne disulfonate (Bcs) were purchased from Sigma and other common commercial sources and were used without further purification. The enzyme rat carboxypeptidase B was purchased from ProSpecBio (Israel). Fluorescent dansyl peptide probes DP1-4 for Cu(II) were synthesized as reported [22]. The concentration of each DP probe was determined by the solution absorbance at 326 nm using the reported absorptivity coefficient $\varepsilon = 4500\ M^{-1}\ cm^{-1}$, common to each probe [22]. A Cu(II) standard solution was prepared by dissolution of $CuSO_4 \cdot 5H_2O$ in Milli-Q water and calibrated via reaction with excess of the Cu(I)-binding ligand Bcs in Mops buffer containing reductant NH_2OH. Under such conditions, all copper ions are converted quantitatively to the well-defined chromophoric complex anion $[Cu^{I}(Bcs)_2]^{3-}$ with characteristic absorbance at 483 nm ($\varepsilon = 13{,}000\ M^{-1}\ cm^{-1}$) [21].

2.2. Construction of expression plasmids

The expression plasmids for CopC variants CopC-H85F and -H1/85F lacking the last two C-terminal amino acid residues (Gly-Gln) were constructed previously [20]. To facilitate protein purification, the expression plasmids were modified by insertion of a DNA sequence coding for three extra lysine residues at the C-terminus of the gene for expression of the equivalent proteins with a tri-lysine tag. For comparison, a plasmid for expression of CopC-H85F with a C-terminal hexa-histidine tag was also constructed by re-cloning the original gene without the stop codon into the pET-20b vector between the restriction sites NdeI and XhoI.

The DNA sequence coding the N-terminal metal binding domain 6 of human Wilson protein (WLN6: the sequence between residues 561–631 of the intact WLN protein) was amplified via PCR with a DNA template containing the intact WLN gene (GenBank U11700.1). This was cloned into a modified pET-20b-$(Lys)_6$ vector between restriction sites NdeI and BamHI to construct an expression plasmid for expression of WLN6 protein with a C-terminal hexa-lysine tag (i.e., WLN6-$(Lys)_6$). The pET-20b-$(Lys)_6$ vector was obtained by insertion of a DNA sequence coding a hexa-lysine fragment between the restriction sites BamHI and HindIII of the original pET-20b plasmid. The plasmid for expression of this tagged version (i.e., WLN6-$(His)_6$) was constructed by cloning the WLN6 gene (without the stop codon) into pET20b vector between the restriction sites NdeI and XhoI. The gene inserts in all expression plasmids were confirmed by DNA sequencing.

2.3. Protein expression and purifications

Each expression plasmid was propagated in *E. coli* Top 10 cells and then transformed into BL21(DE3) CodonPlus cells for protein expression. The transformed cells were grown in 2YT media containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L) in shaking flasks at 37 °C to OD_{600} ~1. Overnight protein expression at ~30 °C was induced with IPTG at a final concentration of 0.4 mM. The cells were harvested by centrifugation.

A clarified cell extract was prepared in Tris–HCl buffer (10 mM; pH 8.0) for each protein except for WLN6-$(Lys)_6$ which was present largely in the lysate pellet. This system required inclusion of ~300 mM NaCl in the buffer for release of the protein into solution. All three poly-lysine tagged proteins were purified readily via a single cation-exchange step. Briefly, for the single mutant protein CopC-H85F-$(Lys)_3$, the clarified cell extract was adjusted to pH ~6 with a minimal volume of Mes solution (~200 mM) and then applied to a CM-52 cation-exchange column (15 × 2.6 cm) pre-equilibrated in Mes buffer (20 mM, pH 6.0). The double mutant protein CopC-H1/85F-$(Lys)_3$, required acetate buffer at pH 5.0 instead of Mes buffer at pH 6.0 to enhance protein binding to the CM-52 resin. The bound target proteins were eluted with a salt gradient of NaCl (0–300 mM) in the same buffers. The protein fractions that contained the target proteins were concentrated and applied to a Superdex 75 gel-filtration column (HR16/60; GE Healthcare) and eluted with Mops buffer (20 mM, pH 7.4; NaCl, 150 mM). An equivalent procedure was followed for the purification of the WLN6-$(Lys)_6$ protein but using Mops buffer at pH 7.0 containing reductant β-mercaptoethanol (5.0 mM) for the cation-exchange step and the reductant tris(2-carboxyethyl)phosphine (TCEP; 0.5 mM) for the gel-filtration elution step. Edta (1.0 mM) was included in the elution buffer for the first step of each purification to ensure removal of contaminating metal ions. The purity and identity of each isolated protein were confirmed by SDS-PAGE and ESI-MS. Protein yields were typically in the range 15–25 mg per 1 L of culture. The estimations were based on the solution absorbance at 280 nm for the purified samples using the calculated extinction coefficients of $\varepsilon = 8{,}480\ M^{-1}\ cm^{-1}$ for CopC and $1{,}490\ M^{-1}\ cm^{-1}$ for WLN6.

The C-terminal lysine tag may be digested readily and completely by incubation with enzyme rat carboxypeptidase B in about 1000:1 M ratio of the tagged protein to the enzyme in 50 mM Mops buffer (pH 7.0) at ambient temperature. The digestion process was monitored via NaCl gradient elution of the digestion mixture from an analytical Mono-S cation-exchange column (1.0 mL) in Mops buffer (20 mM, pH 7.4). The identities of the eluted protein fractions were confirmed by ESI-MS.

For the two hexa-histidine tagged proteins CopC-H85F-$(His)_6$ and WLN6-$(His)_6$, the purification was effective with an immobilized metal-affinity chromatography (IMAC) column preloaded with Ni(II) ions. Briefly, the clarified cell lysate was prepared in buffer A consisting of KP_i (20 mM, pH 7.4), NaCl (300 mM) and imidazole (20 mM) and was loaded onto an IMAC column (~1.0 mL) pre-equilibrated in the same buffer. After the column was washed with a ten-column volume of buffer A, the bound proteins were eluted with buffer B that consisted of buffer A plus 300 mM imidazole. The eluted proteins were concentrated and incubated with 1,2-cyclohexanediaminetetraacetate (CDTA; 1.0 mM) to complex the residual Ni(II) ions and then purified further with the same gel-filtration chromatography described for the other proteins.

The two cysteine side-chains in the three proteins WLN6-$(Lys)_6$ and WLN6-$(His)_6$ comprise a high affinity binding site for Cu(I), but may be oxidized readily to form an intra-molecular disulfide bond. The fully reduced forms were prepared by incubation with dithiothreitol (DTT; 5.0 mM) overnight at 4 °C followed by buffer-exchange in an anaerobic glove-box on a Bio-Gel P-6 DG desalting column (Bio-Rad) to remove the excess reductant. The fully oxidized forms WLN6-ss-$(Lys)_6$ and WLN6-ss-$(His)_6$ were obtained by reaction with 2.2 equiv of $K_3[Fe^{III}(CN)_6]$ in Mops buffer (pH 7.4) for 1 h, followed by the same