Protein Expression and Purification 140 (2017) 1–7

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Expression and purification of single cysteine-containing mutant variants of the mouse prion protein by oxidative refolding

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ARTICLE INFO

Article history:
Received 5 July 2017
Received in revised form
19 July 2017
Accepted 19 July 2017
Available online 21 July 2017

Keywords:
Prion
Cysteine mutant
Oxidative-refolding
Disulfide bond
Thiol labelling

ABSTRACT

The folding and aggregation of proteins has been studied extensively, using multiple probes. To facilitate such experiments, introduction of spectroscopically-active moieties in to the protein of interest is often necessary. This is commonly achieved by specifically labelling cysteine residues in the protein, which are either present naturally or introduced artificially by site-directed mutagenesis. In the case of the recombinant prion protein, which is normally expressed in inclusion bodies, the presence of the native disulfide bond complicates the correct refolding of single cysteine-containing mutant variants of the protein. To overcome this major bottleneck, a simple purification strategy for single tryptophan, single cysteine-containing mutant variants of the mouse prion protein is presented, with yields comparable to that of the wild type protein. The protein(s) obtained by this method are correctly folded, with a single reduced cysteine, and the native disulfide bond between residues C178 and C213 intact. The β-sheet rich oligomers formed from these mutant variant protein(s) are identical to the wild type protein oligomer. The current strategy facilitates sample preparation for a number of high resolution spectroscopic measurements for the prion protein, which specifically require thiol labelling.

1. Introduction

The site-specific incorporation of spectroscopically-active probes into proteins has facilitated a suite of high resolution *in vitro* experiments, spanning a range of length scales [1–3], allowing unprecedented structural characterization of protein structure and dynamics. Many of these probes are introduced into the protein of interest *via* thiol labelling, which additionally demands the purification and characterization of cysteine-containing mutant variants of the wild type (WT) protein. While this is relatively straightforward to do for proteins which lack native disulfide bonds, it can get complicated for proteins which do. The introduction of additional cysteine residues can lead to scrambling and incorrect disulfide bond pairing during expression and purification, resulting in misfolding, aggregation and/or precipitation [4–6].

The problem is further aggravated for proteins which are expressed in inclusion bodies [7,8], where inefficient refolding to their correctly folded native state hinders protein production in quantities typically required for *in vitro* experiments. The development of efficient protocols for the successful refolding of proteins with native disulfide bonds expressed in inclusion bodies in sufficient yields is therefore of great interest [5,9].

The prion protein is an example of such a protein with a native disulfide bond, that is normally expressed in inclusion bodies, and has been refolded and purified in several different ways [10–12]. Purification of the prion protein in a soluble form, though uncommon, has also been reported [13–15]. The prion protein is rich in α-helical content in its native monomeric form, but upon misfolding (under diseased conditions) forms β-sheet rich aggregates [16]. The misfolding and aggregation of the prion protein is responsible for a class of fatal neurodegenerative diseases, together known as spongiform encephalopathies. In contrast to the WT protein, the refolding of cysteine mutant variants of the prion protein has been challenging [17–21]. In the cases where these proteins have been purified, the yields were either too low, or rigorous characterization was not carried out.

The *in vitro* folding [22–25] and aggregation [26–29] of the WT as well as several pathogenic mutant variants of the prion protein [30–32] have been studied extensively. However, experiments which require labelling with spectroscopically-active probes, like paramagnetic nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), fluorescence resonance energy transfer (FRET), fluorescence correlation spectroscopy (FCS) and thiol

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http://dx.doi.org/10.1016/j.pep.2017.07.014
1046-5928/

labelling studies have remained largely unexplored for the prion protein [21,28].

The most common method for the purification of the WT protein employs affinity purification followed by reverse phase chromatography (RPC) [10,11]. When cysteine-containing mutant variants of the mouse prion protein (moPrP) were purified using this protocol, the protein was found to contain multimeric species, with a CD spectrum suggesting significant β-sheet content, unlike the WT protein. Refolding the protein to the native state while it was bound to the affinity column was also tried, but the yields from these preparations were too low for proper characterization and further experiments. It was therefore important to devise a protocol for the proper refolding of the cysteine-containing mutant variants of the prion protein, with sufficient yields and detailed characterization.

Here, a straightforward protocol for the expression and purification of single tryptophan, single cysteine-containing (single Trp, single Cys-containing) mutant variants of the full length moPrP with an intact native disulfide bond, is described. It is demonstrated, using two representative single Trp, single Cys-containing mutant variants of moPrP, W197–C223 and W144–C153 (mouse numbering has been used throughout this manuscript), that this method yields correctly folded highly pure protein in ample quantities, comparable to what has been reported for WT moPrP (Fig. 1). Moreover, the purification protocol has been shown to be applicable for a tryptophan-less (Trp-less) mutant variant which possesses the native disulfide bond, but lacks any additional cysteine residues.

The high refolding yields of single cysteine mutant variants of moPrP will enable many high resolution spectroscopic measurements to be carried out with ease, without the need for complicated and expensive protein production protocols.

2. Materials and methods

2.1. Reagents

All chemicals used for protein purification were purchased from HiMedia and Fisher Scientific, unless otherwise specified. Restriction Enzymes, DNA ligase, Phusion® High-Fidelity DNA Polymerase, dNTPs and *Dpn*I enzyme from New England Biolabs and the DNA miniprep kit from Qiagen were used for molecular biology work. All reagents used for experiments were of the highest purity grade from Sigma Aldrich.

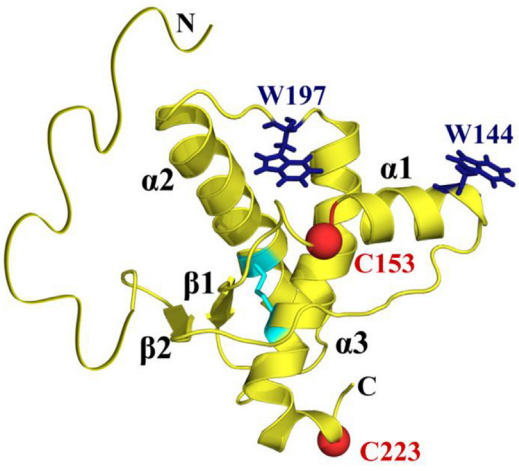

Fig. 1. Design of single Trp, single Cys-containing mutant variants of moPrP. The positions of tryptophans W197 and W144 and cysteines C153 and C223 are shown as blue sticks and red spheres, respectively, mapped on to the structure of the CTD of moPrP (PDB ID 1AG2) [33]. The secondary structural elements and the N- and C-termini are indicated. The disulfide bond between helix 2 and 3 is shown as a cyan stick. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Plasmid construction

The backbone DNA of WT moPrP with all tryptophan residues mutated to phenylalanines was synthesized by GeneScript (USA) in a pUC57 vector. This was subcloned into the pET 22b(+) vector between the *Bam*HI and *Nde*I restriction sites, for expression in *E. coli*. All mutations were made on this backbone, using standard site-directed mutagenesis protocols. First, mutations that would introduce single tryptophan residues were made on the Trp-less background, followed by mutations that would introduce single cysteine residues on these single Trp constructs. The cysteines C178 and C213 already present in the protein, which form the native disulphide bond were left unchanged. All constructs were verified by DNA sequencing before protein expression and purification.

2.3. Protein expression and purification

The pET-22b(+) plasmid encoding the single Trp, single Cys-containing mutant variant, or the Trp-less mutant variant of moPrP, were transformed into *E. coli* BL21(DE3) codon plus (Stratagene) cells. A single colony was used to inoculate 200 ml of LB media containing 100 μg/ml ampicillin, and grown at 37 °C for 8 h. A 50-mL portion of this primary culture was used to inoculate 500 ml of rich media, and grown for 3–3.5 h until it reached an OD_{600} of 1.8–2, when protein expression was induced using IPTG at a final concentration of 0.4 mM. The cells were allowed to grow for 12 h before harvesting and purification. The expressed protein was found to be present in inclusion bodies as described previously [11].

The pellet from 2 L of rich media (~40 g wet cell weight) was resuspended in 100 ml 20 mM Tris, pH 7.8 (Buffer A), and sonicated on ice with a Sonics Vibra-Cell™ Ultrasonic Liquid Processor for a total time of 20 min (5 s "on", 2 s "off") at a power level of 60%. This was centrifuged at 14,000 rpm and 4 °C for 30 min, and the supernatant discarded. The pellet was resuspended in 80 ml of Buffer A, sonicated for another 10 min (using the same settings), and centrifuged at 14,000 rpm and 4 °C for 30 min. The supernatant was discarded, and the pellet containing the inclusion bodies was solubilized in 80 ml of 6 M guanidinium hydrochloride (GdnHCl), 20 mM Tris, 1 mM reduced glutathione (GSH), pH 7.8 (Buffer G), and sonicated for a final 10 min. The pellet was disrupted manually with a glass rod, in between rounds of sonication to aid in solubilisation of the inclusion bodies. This was then centrifuged at 14,000 rpm and 4 °C for 45 min, and the supernatant containing the denatured protein was collected.

The supernatant was added to 30 ml of Ni Sepharose 6 Fast Flow beads (GE Healthcare), charged with nickel sulphate and equilibrated with Buffer G, and mixed by shaking on a rocker at room temperature (RT) for an hour with intermittent manual mixing. The equilibrated mixture was loaded in to a Vensil® glass column, and washed with 800 ml of Buffer G. The protein was finally eluted in 50 ml Buffer E (Buffer G + 200 mM imidazole, pH 7.8). The eluate was first dialyzed against 2 L of Buffer A containing 3 M GdnHCl and 1 mM GSH for 12 h followed by another round of dialysis against 2 L of Buffer A containing 1 M GdnHCl and 1 mM GSH for 12 h at 4 °C. Finally, to allow for correct disulfide formation, 0.06 g of oxidized glutathione (GSSG) was added to the protein solution (typically ~50 ml) to a final concentration of 0.2 mM, and stirred overnight at 4 °C. The protein was then dialyzed against 5 L of Buffer A (3 changes) to completely remove GdnHCl, GSH and GSSG. Some precipitation was seen at this stage, possibly due to misfolding and