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Smart polymer mediated purification and recovery of active proteins from inclusion bodies

Saurabh Gautam[a], Priyanka Dubey[a], Pranveer Singh[b], S. Kesavardhana[b], Raghavan Varadarajan[b], Munishwar N. Gupta[a,*]

[a] Department of Chemistry, Indian Institute of Technology Delhi, Hauz Khas, New Delhi 110016, India
[b] Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India

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ABSTRACT

Obtaining correctly folded proteins from inclusion bodies of recombinant proteins expressed in bacterial hosts requires solubilization with denaturants and a refolding step. Aggregation competes with the second step. Refolding of eight different proteins was carried out by precipitation with smart polymers. These proteins have different molecular weights, different number of disulfide bridges and some of these are known to be highly prone to aggregation. A high throughput refolding screen based upon fluorescence emission maximum around 340 nm (for correctly folded proteins) was developed to identify the suitable smart polymer. The proteins could be dissociated and recovered after the refolding step. The refolding could be scaled up and high refolding yields in the range of 8 mg L^{-1} (for CD4D12, the first two domains of human CD4) to 58 mg L^{-1} (for malETrx, thioredoxin fused with signal peptide of maltose binding protein) were obtained. Dynamic light scattering (DLS) showed that polymer if chosen correctly acted as a pseudochaperonin and bound to the proteins. It also showed that the time for maximum binding was about 50 min which coincided with the time required for incubation (with the polymer) before precipitation for maximum recovery of folded proteins. The refolded proteins were characterized by fluorescence emission spectra, circular dichroism (CD) spectroscopy, melting temperature (T_m), and surface hydrophobicity measurement by ANS (8-anilino1-naphthalene sulfonic acid) fluorescence. Biological activity assay for thioredoxin and fluorescence based assay in case of maltose binding protein (MBP) were also carried out to confirm correct refolding.

1. Introduction

While it is well established that a correctly folded conformation of a protein – called the native structure – is responsible for its biological activity [1], the exact mechanisms are still less than completely understood. The well known work by Anfinsen showed that the information for folding resides in the primary sequence of the protein [2]. As Hartl et al. [3] recently observed "Although small proteins may fold at very fast speeds (within microseconds), in dilute buffer solutions, larger multidomain proteins may take minutes to hours to fold, and often even fail to reach their native states in vitro". In vivo, protein crowding [4] contributes to aggregation of non-native structures. This is prevented by molecular chaperones or chaperonins in a cell. Their role is not always limited to prevention of aggregation, but may extend to acceleration of folding and reversal of misfolding events [3]. Many excellent reviews are available on protein folding [5,6]. While Sinha and Udgaonkar [5] have provided a rigorous treatment of early events in protein folding, Nickson and Clarke [6] have reviewed both theoretical and experimental methods (and their results) used to study protein folding. There is enough evidence that protein folding involves existence of one or more partially folded structures. In many cases, it is possible to isolate 'molten globules' which occur on the folding pathway. The 'oil drop' model of protein structure envisages that there is a hydrophobic core with polar amino acids on the surface H-bonded with water. Hydrophobic clusters do occur on the protein surface and are quite often part of a specific binding site for ligands/substrates. Apart from the above 'nucleation model', 'energy landscape model' has also been proposed more recently, where folding intermediates are viewed as 'kinetic traps' on the folding pathway. The greater understanding of protein folding is also of practical utility in the context of protein refolding. The overexpression of recombinant proteins in bacterial hosts often leads to the formation of inactive and insoluble aggregates called inclusion bodies. In some cases, proteins in these inclusion bodies may not be completely inactive [7]. Protein aggregation as such has also attracted attention as the cause behind several neurodegenerative diseases and cataract formation

* Corresponding author. Tel.: +91 11 2659 1503; fax: +91 11 2658 1073.
E-mail address: appliedbiocat@yahoo.co.in (M.N. Gupta).

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[8]. From the biotechnological perspective, the recovery of soluble active proteins from inclusion bodies generally involves: (1) solubilization of the inclusion bodies by denaturants to obtain the protein in an unfolded form, (2) a second step of refolding [9–11]. While a large number of strategies have been described in the literature for the refolding step [9,11–14], it is a "no single shoe fits all" situation.

Tsumoto et al. [12] have provided an excellent overview of different classes of additives which have been used during protein refolding. These can be classified as folding enhancers (e.g. sucrose, ammonium sulfate) or aggregation suppressors (e.g. mild denaturants or low concentration of denaturants like urea or guanidine hydrochloride). The use of PEG in assisting protein refolding is a pioneering study in the area. While its detailed role on the basis of thermodynamics has been discussed [15], it essentially binds to hydrophobic regions of the folding intermediate(s), prevents protein–protein interaction and hence essentially works as an aggregation suppressor. Use of smart polymers as "pseudochaperonins" for refolding has been described by various workers as one such approach [16–19]. Smart polymers are reversibly soluble–insoluble materials which respond to various stimuli such as pH changes, temperature changes and presence of different chemical species [20–25]. Use of smart polymers (as compared to other water soluble polymers like PEG) offers the added advantage that one can easily isolate the folded protein–polymer complex as a precipitate by applying a suitable stimulus. This precipitation often will also result in simultaneous purification of the desired protein [21,26,27]. In the past, refolding by smart polymers has been generally limited to working with chemically or thermally denatured proteins [16,28,29]. A few years back, we reported use of a pH-responsive methyl methacrylate polymer for obtaining active recombinant controller of cell division or death B (CcdB) protein from its inclusion bodies [19]. Unfortunately, refolded CcdB could not be dissociated from that polymer Eudragit S-100.

In this work, we have attempted to develop the use of smart polymers as a general approach for recovery of active proteins from their inclusion bodies. Obviously, a single smart polymer would not work with different proteins. So, we decided to develop a screen for the searching of a suitable smart polymer in a 96-well plate format. Our results show that, at least with a variety of proteins investigated by us, it was possible to identify a suitable smart polymer for obtaining a reasonable recovery of the active protein by refolding. The refolding was confirmed by biological activity (wherever possible), fluorescence, and circular dichroism (CD) spectroscopy. The proteins used for refolding from inclusion bodies in the present study are: five aggregation prone mutants of the *E. coli* proteins-controller of cell division or death B (CcdB) [30], maltose binding protein (MBP) [31], and thioredoxin fused with signal peptide of MBP (malETrx) [13]; the first two domains of human CD4 (CD4D12) [32]; single chain variable fragment (ScFv) b12 and single chain antigen binding fragment (ScFab) b12, both derived from the anti-human immunodeficiency virus (HIV)-1 antibody b12, which binds to the CD4 binding site on gp120 of HIV-1 [33].

2. Materials and methods

2.1. Materials

Eudragit L-100 and S-100 were products of Rohm Pharma GmbH (Weiterstadt, Germany). This is a copolymer of methacrylic acid and methyl methacrylate (in a molar ratio of 1:1) with average molecular weight of 1,35,000 g/mol (Product sheet, Rohm Pharma). Cationic starch (Catamyl-VS; 99.6% purity), starch derivatized with a quaternary ammonium compound, was a kind gift from Chemtech Marketing (Delhi, India). Protanal LF 10/60 (free alginate from brown seaweed) having a high content of guluronic acid (65–75%) was a product of Protan A/S (Drammen, Norway). The average molecular weight of Protanal LF 10/60 is 3,20,000 g/mol [34]. Alginic acid, composed predominantly of mannuronic acid residues (catalog no. A-2158), 8-anilino1-naphthalene sulfonic acid (ANS), phenylmethanesulfonylfluoride (PMSF), isopropyl β-D-thiogalactopyranoside (IPTG) and ampicillin were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Strains and expression plasmids

E. coli BL21 (DE3) was used for protein expression of malETrx, human CD4D12, mutants of MBP, ScFv b12 and ScFab b12. *E. coli* CSH501 was used for expressing wild type (WT) CcdB and its mutants. The plasmids used for expression of these proteins were pBAD24 containing CcdB-F17P, CcdB-M97K, MBP224D and MBP264D inserts, pET20b(+) containing (A14E)malETrx insert, pET28a containing human CD4D12 insert, pET22(+) containing ScFv b12 insert and pComb containing ScFab b12 insert.

2.3. Overexpression in E. coli

The plasmid pBAD24 expressing CcdB mutants F17P or M97K, was transformed into *E. coli* CSH501 [30]. A single colony was picked and inoculated into 5 mL LB medium containing 100 μg mL^{-1} ampicillin. One percent of primary inoculum was transferred into 1 L fresh LB broth (amp^{+}) and grown at 37 °C with shaking at 200 rpm until OD_{600} reached 0.8. Induction was carried out by adding L-arabinose (0.2%) and the culture was further grown under similar conditions for 12 h at 37 °C at 200 rpm. This procedure was repeated for the transformation of the plasmid pET20b(+) containing (A14E) malETrx insert (showing leaky expression), pBAD24 containing MBP 224D and 264D inserts, pET22(+) containing ScFv b12 insert and pComb containing ScFab b12 insert into *E. coli* BL21 (DE3). The plasmid pET28a expressing CD4D12 was transformed into *E. coli* BL21 (DE3) and 50 μg mL^{-1} kanamycin was used as the selection marker. Induction was carried out by adding L-arabinose (0.2%) in case of MBP224D and MBP264D; 0.5 mM IPTG (final concentration) in case of malETrx and CD4D12; and 1 mM IPTG (final concentration) in case of ScFv b12 and ScFab b12, and the culture was further grown under similar conditions for 12 h.

2.4. Isolation and solubilization of inclusion bodies

Cells were harvested, sonicated in resuspension buffer (For CcdB mutants, 50 mM Tris/pH 8.0/1 mM EDTA/10% glycerol/200 mM PMSF; for malETrx, MBP mutants, ScFv b12 and ScFab b12, 50 mM Tris/pH 7.0/150 mM NaCl/1 mM EDTA/100 mM PMSF; for CD4D12, PBS/pH 7.4/100 mM PMSF) 10 times with 30 s pulses on ice, and centrifuged at 9000 × *g* for 30 min at 4 °C. The inclusion body pellet was washed (thrice) with washing buffer (50 mM PBS/pH 7.4/0.5% Triton X-100) and centrifuged at 9000 × *g* for 30 min. Isolated inclusion bodies were solubilized with 8 M urea in 50 mM Tris–HCl buffer (pH 7.5 for CcdB mutants, malETrx, CD4D12 and MBP mutants and pH 7.0 for ScFv b12 and ScFab b12) containing 100 mM DTT and incubated with stirring for 5 h at room temperature.

2.5. Preparation of Eudragit solution

Eudragit solutions (2%, w/v) for both kinds of Eudragit (L-100 and S-100) were prepared by suspending the polymer powder (2 g) in 50 mL of 50 mM Tris–HCl buffer, pH 7.5. The pH of the solution was raised to 11.0 with 3 M NaOH and stirred until the polymer dissolved. The pH was then readjusted to 7.5 with 3 M HCl, and the volume of the solution was increased to 100 mL with buffer.