Simultaneous refolding and purification of recombinant proteins by macro-(affinity ligand) facilitated three-phase partitioning

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ABSTRACT

A strategy called macro-(affinity ligand) facilitated three-phase partitioning (MLFTPP) is described for refolding of a diverse set of recombinant proteins starting from the solubilized inclusion bodies. It essentially consists of: (i) binding of the protein with a suitable smart polymer and (ii) precipitating the polymer-protein complex as an interfacial layer by mixing in a suitable amount of ammonium sulfate and t-butanol. Smart polymers are stimuli-responsive polymers that become insoluble on the application of a suitable stimulus (e.g., a change in the temperature, pH, or concentration of a chemical species such as Ca²⁺ or K⁺). The MLFTPP process required approximately 10 min, and the refolded proteins were found to be homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The folded proteins were characterized by fluorescence emission spectroscopy, circular dichroism spectroscopy, biological activity, melting temperature, and surface hydrophobicity measurements by 8-anilino-1-naphthalene-sulfonate fluorescence. Two refolded antibody fragments were also characterized by measuring Kᵦ by Biacore by using immobilized HIV-1 gp120. The data demonstrate that MLFTPP is a rapid and convenient procedure for refolding a variety of proteins from inclusion bodies at high concentration. Although establishing the generic nature of the approach would require wider trials by different groups, its success with the diverse kinds of proteins tried so far appears to be promising.

Macro-(affinity ligand) facilitated three-phase partitioning (MLFTPP) is a relatively underexploited technique for bioseparation of proteins [1–3]. It essentially involves precipitation of a smart polymeric affinity ligand in the presence of the target protein. The precipitation is carried out by mixing in appropriate amounts of ammonium sulfate and t-butanol. The precipitation of the affinity complex takes place at the interface of upper t-butanol rich phase and lower water-rich phase. In the current work, MLFTPP has been successfully shown to refold a diverse set of recombinant proteins starting from their inclusion bodies.

Inclusion bodies are an insoluble and inactive form of recombinant proteins that are often obtained during overexpression in bacterial hosts. Recovery of active proteins from these inclusion bodies generally requires a solubilization step using urea or similar denaturing agents [4,5]. This is followed by a refolding step. Numerous strategies for protein refolding have been described [4–12]. It is often a matter of hit and trial because no general method is available. Hence, the development of newer methods for refolding is considered as desirable. We recently described the application of affinity precipitation (AP) for refolding [13]. To obtain a fair comparison, the set of proteins chosen in the current work was identical to the earlier work with AP. All of these proteins differ in their molecular weights, isoelectric points, number of disulfide bonds, and the like.

Both techniques (AP and MLFTPP) use a smart polymer to create a smart polymeric affinity ligand. Smart polymers chosen for this application are reversibly soluble-insoluble polymers that precipitate on the application of an appropriate stimulus that may include a change in the pH or temperature or the addition of a chemical species such as Ca²⁺ or K⁺, depending on the individual smart polymer [14]. Both naturally occurring polymers (e.g., alginate, chitosan, κ-carrageenan) and synthetic polymers (e.g., methylmethacrylates) have been used for direct precipitation of proteins in AP [13] and MLFTPP [1,2]. The original concept was that a suitable affinity ligand can be linked to a specific smart polymer to convert it into a smart polymeric affinity ligand. In both
techniques, an affinity complex between the target protein and smart polymeric affinity ligand is formed in free solution. This is similar to what happens in the widely used technique of affinity chromatography [15] except that in affinity chromatography an affinity ligand is linked to an insoluble polymer and, hence, the affinity complex is not formed in free solution. The merits and demerits of chromatographic and nonchromatographic approaches in protein separation have been adequately discussed in the literature [3,16]. In AP, the affinity complex is precipitated by an appropriate stimulus depending on the smart polymer component. In MLFTPP, the precipitation is caused by mixing in salt and organic solvent. The precipitate is formed as the interfacial layer. Until this step, one does not need a smart polymer for MLFTPP. However, in the next step, in both AP and MLFTPP, the affinity complex precipitate is dissolved in a dissociating buffer. Dissociation of the complex into protein and (smarter) macroaffinity ligand occurs because of the presence of either salt or ethylene glycol or competitive inhibitor in the buffer. Now, an appropriate stimulus is applied to reprecipitate just the macroaffinity ligand, and the desired protein is retained in the supernatant. It is at this stage that MLFTPP requires the polymeric compound to be a smart polymer rather than just a water-soluble polymer.

Many years back, Sharma and coworkers [2] showed that MLFTPP could refold a urea denatured pectinase. Hence, the current work was undertaken to see whether MLFTPP as a refolding method would work starting with inclusion bodies as well and also to improve on AP as a refolding strategy. MLFTPP offers the following potential advantages over AP [13]:

(i) Whereas in AP the precipitation of the polymer required an appropriate stimulus (depending on the nature of the smart polymer), in MLFTPP the precipitation is caused by mixing in appropriate amounts of ammonium sulfate and t-butanol (irrespective of the nature of the polymer). Admittedly, the amount of ammonium sulfate and ratio (v/v) of t-butanol to initial aqueous phase needs to be optimized with each polymer. Luckily, the range of optimal values for either of these variables was found to be quite narrow [2,17].

(ii) With AP, the time taken is approximately 1 h [13,18]. We show in the current work that MLFTPP takes approximately 10 min, which is a much shorter process time.

(iii) Although refolding a dilute solution of protein is not difficult, obtaining refolded proteins directly at a reasonable concentration continues to be a worthwhile goal. MLFTPP is also essentially a precipitation technique (i.e., the end result is a highly concentrated form of the protein). It is shown in the current work that MLFTPP yields a precipitate that is less hydrated than the one obtained in the case of AP. This not only results in a refolded protein in a more concentrated form but also has a greater potential for better purification. This is explained further during the discussion of the results.

Over the years, it has been observed that many smart polymers as such display inherent “affinity” for a given protein. Hence, when added to a mixture of proteins, an appropriately chosen smart polymer would selectively bind to the given protein. A screen in a 96-well plate format was recently described for choosing an appropriate smart polymer for a given protein [13]. Why do smart polymers show this selectivity? The smartness originates in the presence of diverse monomers. The diversity in their structure confers on these polymers the capability of interacting with the proteins via multiple weak interactions [14]. Multiple weak interactions are the basis of molecular recognition in both chemistry and biology. Hence, smart polymers are just another addition to the list of affinity ligands such as metal chelates, dyes, peptides, and aptamers that have no in vivo relationship with the protein [15,19]. This, of course, also means that MLFTPP, apart from refolding, also leads to simultaneous purification.

Materials and methods

Overexpression in E. coli

Overexpression of recombinant proteins in Escherichia coli was carried out as described previously [13]. The plasmid pBAD24 expressing CcdB (controller of cell division or death B protein) mutants F17P and M97K was transformed into E. coli CSH501. A single colony was picked and inoculated into 10 ml of LB medium containing 100 μg ml⁻¹ ampicillin. Then 1% of primary inoculum was transferred into 1 L of fresh LB broth (amp⁻) and grown at 37 °C with shaking at 200 rpm until OD₆₀₀ 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 with shaking at 200 rpm. This procedure was repeated for the transformation of the plasmid pgFPuv-containing recombinant GFP (green fluorescent protein) insert, pET20b(+) containing (A14E)malETrx insert (showing leaky expression), pBAD24-containing MBP224D and MBP264D inserts, pET22b(+) 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 isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration) in case of CD4D12 and malETrx, and 1 mM IPTG (final concentration) in case of recombinant GFP, ScFv b12, and ScFab b12, and the culture was further grown under similar conditions for 12 h.

Isolation of overexpressed GFP from E. coli

The cells were harvested by centrifugation at 8000g for 10 min at 4 °C. GFP was isolated from E. coli cells by sonication in 0.05 M phosphate buffer (pH 7.5) containing 2 M NaCl and 100 μM phenylmethanesulfonyl fluoride (PMSF), three times with 15-s pulses on ice, and centrifugation at 9000g for 10 min at 4 °C. The supernatant obtained in this way was used as a crude extract for GFP.

Isolation and solubilization of inclusion bodies

Inclusion bodies for all of the proteins were isolated as described previously [13]. Isolated inclusion bodies were solubilized with 8 M urea in 50 mM Tris–HCl buffer (pH 7.5) for CcdB mutants, CD4D12, malETrx, and MBP (maltose binding protein) mutants and pH 7.0 for ScFv b12 and ScFab b12) containing 100 mM dithiothreitol (DTT) and incubated with stirring for 5 h at room temperature.

Preparation of solutions of smart polymers

Solutions of Eudragit L-100 solution (2%, w/v), cationic starch (2%, w/v), and Protranal LF alginate (5%, w/v) were prepared as described previously [13].

Optimization of MLFTPP conditions for Eudragit L-100

The varying concentrations of Eudragit L-100 (2 ml) in 50 mM Tris–HCl (pH 7.5) were mixed with varying concentrations of ammonium sulfate (w/v) and t-butanol (v/v). The mixture was centrifuged at 3000g for 5 min at 25 °C for three-phase formation. Three phases were formed—the upper organic phase, an interfacial precipitate containing Eudragit L-100, and the lower aqueous
phase—and were collected separately. The precipitated Eudragit L-100 was suspended in 2 ml of distilled water, its pH was adjusted to 4.0 with 2 M acetic acid, and the turbidity was read at 470 nm [20]. The amount of Eudragit L-100 precipitated was calculated by taking the turbidity obtained with the starting amount of Eudragit L-100 as 100%.

Refolding of CcdB mutants (CcdB-F17P and M97K), human CD40D12, and malETrx from inclusion bodies

The solubilized inclusion bodies (1.5 ml) were added to 0.5 ml of 2% (w/v) Eudragit L-100 (0.5%, w/v, final concentration). The final protein concentration was 1.0–3.0 mg ml⁻¹. The solution was then subjected to MLFTPP by saturating it with ammonium sulfate (30%, w/v) and vortexed gently to dissolve the salt. Following this, t-butanol was added in a 1:1 (v/v) ratio of the solution to t-butanol. After the mixture was centrifuged at 3000g for 5 min at 25 °C, three phases were formed—the upper organic phase, an interfacial precipitate containing Eudragit L-100-bound target protein, and the lower aqueous phase—and were collected separately. The polymer-bound target protein (interfacial precipitate) was dissociated from the polymer by adding 70% (v/v) ethylene glycol solution made in 50 mM Tris–HCl buffer (pH 7.5) and incubating for 1 h at 4 °C with shaking at 150 rpm. Dissociated protein was recovered by precipitating Protanal LF with 1 M CaCl₂ (final concentration of CaCl₂ in solution was 60 mM) [13]. The precipitated polymer was separated from the supernatant by centrifugation (10,000g, 10 min, 4 °C). Further characterization and activity assays were determined in the supernatant after extensive dialysis against the 50 mM Tris–HCl buffer (pH 7.0) to remove NaCl.

Refolding of MBP mutants (224D and 264D) from inclusion bodies

The solubilized inclusion bodies (1.7 ml) were added to 0.3 ml of 2% (w/v) cationic starch (0.3%, w/v, final concentration). The final protein concentration was 1.0–3.0 mg ml⁻¹. The solution was then subjected to MLFTPP by saturating it with ammonium sulfate (30%, w/v) and vortexed gently to dissolve the salt. Following this, t-butanol was added in a 1:2 (v/v) ratio of the solution to t-butanol [2]. After the mixture was centrifuged at 3000g for 5 min at 25 °C, three phases were formed: the upper organic phase, an interfacial precipitate containing cationic starch-bound protein, and the lower aqueous phase. The polymer-bound target protein (interfacial precipitate) was dissociated from polymer by suspending the polymer–protein complex in 2 ml of chilled 1 M NaCl (prepared in 50 mM Tris–HCl buffer, pH 7.0) and incubating this suspension at 4 °C for 18 h with shaking at 150 rpm. Dissociated protein was recovered by precipitating Protanal LF with 1 M CaCl₂ (final concentration of CaCl₂ in solution was 60 mM) [13]. The precipitated polymer was separated from the supernatant by centrifugation (10,000g, 10 min, 4 °C). Further characterization and activity assays were determined in the supernatant after extensive dialysis against the 50 mM Tris–HCl buffer (pH 7.0) to remove NaCl.

PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the protein samples using 15% gel was performed according to Hames [22] using a Genei gel electrophoresis unit (Bangalore Genei, Bangalore, India). The protein bands on the gel were visualized using Coomassie blue stain.

Estimation of protein concentration

The protein concentrations were estimated using extinction coefficient values of 16,100 M⁻¹ cm⁻¹ at 278 nm in case of CcdB [23], 18,450 M⁻¹ cm⁻¹ at 280 nm for CD40D12 as calculated from the amino acid sequence [24], and 1.46 mg ml⁻¹ cm⁻¹ at 280 nm for MBP [21]. The protein concentration in other cases was estimated by the dye binding method using bovine serum albumin as the standard protein as described previously [25].

Fluorescence measurements

All fluorescence spectra were recorded at 25 °C on a Varian Cary Eclipse spectrofluorimeter (Varian, Mulgrave, Victoria, Australia). Typically, 1.0–2.0 μM protein in 10 mM Tris–HCl (pH 7.5) was used, and the fluorescence emission spectra were recorded from 300 to 400 nm on excitation at 280 nm. All 8-anilino-1-naphthalenesulfonate (ANS) binding fluorescence measurements were carried out by using protein and ANS concentrations of 1 and 100 μM, respectively, with excitation at 390 nm, and emission spectra were collected over the wavelength range of 420–600 nm. The excitation and emission slit widths were kept at 2 and 5 nm, respectively. All fluorescence spectra were normalized and corrected for buffer contributions.

CD measurements

Far-ultraviolet (UV) circular dichroism (CD) spectra were recorded on a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) at 25 °C using a cell with a path length of 0.1 cm. Typical spectral accumulation parameters were a scanning rate of 50 nm/min with a 1-nm bandwidth over the wavelength range of 200–250 nm with six scans averaged for each far-UV spectrum. The CD data are presented in terms of mean residue ellipticity (MRE) as a function of wavelength, calculated according to the procedure described previously [26] using a protein concentration of 10–15 μM in 10 mM Tris–HCl (pH 7.5). All CD spectra were corrected for buffer contributions.

Thermal denaturation curves were determined directly by monitoring the ellipticity changes at 222 nm [13].
Dynamic light scattering (DLS) measurements were performed at 25 °C using the instrument laser–spectroscattering 201 by RINa (Berlin, Germany). Data analysis was done using PMgr (version 3.01p17) software supplied with the instrument. Solubilized inclusion bodies (with a protein concentration of 0.5 mg ml–1) in 10 mM Tris–HCl buffer (pH 7.5) were incubated with smart polymer for different time intervals at 25 °C in the presence or absence of 30% (w/v) ammonium sulfate. A sample volume of 50 μl was manually injected into a flow cell (1.5-mm path length) and illuminated by a 100-mW, 660-nm laser diode. Prior to measurements, the buffer solutions were filtered through a 0.2-μm filter, whereas the smart polymer solution was centrifuged at 4000 g for 15 min and the solubilized inclusion bodies were centrifuged at 10,000 g for 15 min before use.

Gyrase inhibition assay for CcdB proteins

The gyrase inhibition assay was carried out by a similar protocol as described previously [27]. The reaction mixtures containing 2 U of E. coli DNA gyrase in gyrase assay buffer (35 mM Tris [pH 7.5], 24 mM KC1, 6 mM MgCl2, 1.8 mM spermidine, 0.36 mg ml–1 BSA, and 1.4 mM ATP) were incubated with CcdB proteins (wild-type [WT]-CcdB and MLFTPP-refolded CcdB mutants at a concentration of 75 ng ml–1) at 25 °C for 10 min. Aliquots containing 0.4 μg of relaxed pUC19 plasmid were added to the reaction mixtures, and the reaction mixtures were further incubated at 25 °C for 1 h. At the end of 1 h, the reaction was terminated by adding 8 μl of 5× dye mix (5% SDS, 25% glycerol, and 0.25 mg ml–1 bromophenol blue), and the products were analyzed by electrophoresis on a 0.8% agarose gel run overnight at 2 V/cm. Gels were stained in TBE (Tris, borate, and EDTA [ethylenediaminetetraacetic acid] buffer) containing 0.5 μg ml–1 ethidium bromide for 1 h and destained for 1 h in TBE.

Insulin aggregation assay for thioredoxin

Thioredoxin aggregation was assayed for its activity by the insulin aggregation assay as described previously [28].

Maltose binding assay for MBP

The binding of maltose to MBP was assayed fluorimetrically by observing a red shift and quenching in the intrinsic tryptophan fluorescence of MBP on maltose binding [29].

SPR experiments

All surface plasmon resonance (SPR) experiments were performed with a Biacore 2000 (Biacore, Uppsala, Sweden) optical biosensor at 25 °C. Full-length HIV-1 gp120 was attached by standard amine coupling to the surface of a research-grade CM5 chip. HIV-1 gp120 was derived from Bal strain (subtype B). A sensor surface (without gp120 or any antibody) that had been activated and deactivated served as a negative control for each binding interaction. Different concentrations of WT gp120 or ScFv b12 and ScFab b12 were run across each sensor surface in a running buffer of phosphate-buffered saline (PBS, pH 7.4) containing 0.01% P20 surfactant. For ScFv b12 protein concentrations taken were 245 nM to 1.86 μM, whereas for ScFab b12 they ranged from 925 nM to 1.23 μM. Both association and dissociation were measured for 300 s. In all of the cases, the sensor surface was regenerated between binding reactions by one or two washes with 10 mM HCl for 30 s at 30 μl/min and with 10 mM NaOH for 30 s at 30 μl/min. Each binding curve was corrected for nonspecific binding by subtraction of the signal obtained from the negative control flow cell. The kinetic parameters were obtained by fitting the data to a simple 1:1 Langmuir interaction model by using BIA Evaluation 3.1 software.

Results and discussion

MLFTPP

The basic design of MLFTPP is illustrated in Fig. 1 by using GFP as a model protein for better visualization of the process. Eudragit L-100, a commercially available polymethylmethacrylate [20], was fortuitously found to bind to GFP. Our experience so far [13,30] has shown that methylmethacrylates such as Eudragit are very useful for selective binding to many proteins. Hence, binding of GFP to Eudragit L-100 was not very surprising. A solution of GFP mixed with an appropriate amount of Eudragit L-100 and ammonium sulfate, when further mixed with t-butanol, led to the precipitation of GFP-bound Eudragit L-100 as an interfacial layer (Fig. 1). The compact nature of this precipitate layer indicates that MLFTPP as a refolding method has an inbuilt concentration step. Recently, we reported that direct precipitation of a smart polymer–protein complex by an appropriate stimulus (the stimulus would vary from polymer to polymer) could also refold a diverse set of proteins [13]. Such precipitates characterized earlier have been found to be considerably hydrated, for example, the precipitation of Eudragit was found to trap approximately 31 g of water per gram of dry Eudragit [31]. This can result in physical trapping of contaminating proteins in a nonspecific fashion. This, in turn, would lead to a refolded protein preparation that might not be homogeneous. Hence, the highly compact nature of the interfacial layer of polymer–protein in MLFTPP can turn out to be a great advantage in some cases. The results reported later in this article with CcdB-F17P, CD4D12, and ScFv b12 show that MLFTPP indeed has greater capacity for simultaneous purification than AP. In addition, unlike precipitation by using stimuli–responsive polymers, a general strategy is possible because precipitation is by the common presence of ammonium sulfate and t-butanol.

Optimization of MLFTPP conditions for Eudragit L-100

The three parameters that are required to be optimized in MLFTPP are amount of ammonium sulfate (w/v), polymer concentration (w/v), and ratio of t-butanol to the solution of the solubilized inclusion bodies with the smart polymer and ammonium sulfate (Table 1). It was seen that maximum Eudragit L-100 precipi-
E. coli both mutants form inclusion bodies during overexpression in substantially buried in the wild type. Hence, it is not surprising that both cases, mutations were carried out at residues that are sub-

100.

of the solubilized inclusion bodies in the presence of Eudragit L-

Eudragit L-100[13] and could be refolded by carrying out MLFTPP with the polymer[13].

used as a refolding strategy as compared with direct precipitation mixing the smart polymer solution with the solubilized inclusion bodies in the MLFTPP protocol), it would happen instantaneously after

is carried out in the presence of ammonium sulfate (as happens in both cases[18,21]. It is interesting to observe that DLS showed that the presence of 30% ammonium sulfate led to protein binding instantaneously

with the polymers. The requirement of approximately 50 min for protein–polymer complex precipitates in a higher amount than polymer alone[32]. Hence, 94% precipitation of the polymer was found to be an acceptable level to proceed further. It may be noted that under the optimized conditions, the protein alone did not precipitate in the interfacial layer (data not shown). For example, to precipitate CcdB-F17P alone required a different set of conditions [9]. Hence, the proteins obtained in the interfacial layer were present as part of a complex with the polymer. Light scattering data (Fig. S1 of the supplementary material) indicated that such binding did indeed take place.

Kinetics of binding of CcdB-F17P and ScFv b12 proteins with smart polymers

The inclusion bodies of two recombinant proteins CcdB-F17P and an antibody fragment ScFv b12 were used to gain insight into interaction of denatured proteins with the smart polymers during MLFTPP (Fig. S1). In both cases, DLS data showed that the presence of 30% ammonium sulfate led to protein binding instantaneously with the polymers. The requirement of approximately 50 min for such binding in the absence of ammonium sulfate is known in several cases [18,21]. It is interesting to observe that DLS showed that in both cases, binding of the polymer in the presence of ammonium sulfate resulted in larger sizes of the protein–polymer complexes. Although these results were obtained in solution, the implication was that if precipitation of protein–polymer complex is carried out in the presence of ammonium sulfate (as happens in the MLFTPP protocol), it would happen instantaneously after mixing the smart polymer solution with the solubilized inclusion bodies. This resulted in a shorter process time where MLFTPP is used as a refolding strategy as compared with direct precipitation with the polymer [13].

Refolding of CcdB mutants (CcdB-F17P and M97K), human CD4D12, and malETrx from inclusion bodies

CcdB-F17P, M97K, CD4D12, and malETrx are known to bind to Eudragit L-100 [13] and could be refolded by carrying out MLFTPP of the solubilized inclusion bodies in the presence of Eudragit L-100.

CcdB is a homodimer and consists of 101 amino acids. It is a cysteotoxin in E. coli because it inhibits DNA gyrase [33–35]. The mutants M97K and F17P have been characterized previously [36]. In both cases, mutations were carried out at residues that are substantially buried in the wild type. Hence, it is not surprising that both mutants form inclusion bodies during overexpression in E. coli [36–38].

Both CcdB mutants refolded by MLFTPP were found to be biologically active, as shown by the inhibition of the DNA supercoiling activity of the E. coli DNA gyrase (Fig. 3). We reported previously [30] that refolding of CcdB-F17P by AP with Eudragit S-100 led to a preparation with which DNA gyrase inhibition assay could not be carried out, presumably due to contamination with nuclease. With MLFTPP, these results on DNA gyrase inhibition assay with CcdB-F17P showed that the protein refolded by MLFTPP had greater purity and was devoid of any nuclease contamination. The intrinsic fluorescence emission spectra showed a maximum at 340 nm in native buffer for both mutants just like WT-CcdB (see Fig. S2A of the supplementary material), indicating that MLFTPP has refolded the mutants with a conformation similar to the native form. The far-UV CD spectra of the two mutants, CcdB-F17P and M97K refolded by MLFTPP, were similar to that of WT-CcdB (see Fig. S2B), suggesting that the secondary structure contents of the refolded CcdB mutants were similar to the native WT-CcdB. ANS binding measurements with the MLFTPP-refolded CcdB-F17P and CcdB-M97K showed similar ANS binding at pH 7.0 (Fig. S2C) and pH 4.0 (Fig. S2D) just like WT-CcdB [39], suggesting native-like structures of these MLFTPP-refolded CcdB mutants.

CD4D12 was also refolded by MLFTPP, and the refolded protein was characterized by intrinsic fluorescence emission spectra, CD spectra, and ANS fluorescence (see Fig. S3 of supplementary material). All of these structural characteristics were similar to the folded structure of CD4D12 described previously [40]. It may be mentioned that CD4D12 previously was refolded by three-phase partitioning (TPP) [9]. In that case, an additional ultrafiltration step was required to obtain a pure preparation. In the current work, refolding of the same protein carried out with MLFTPP did not require this additional step to obtain a pure refolded CD4D12 (Fig. 4A).

The malETrx was purified and refolded by MLFTPP from its inclusion bodies and characterized by intrinsic fluorescence, far-UV CD spectra, and insulin aggregation assay. malETrx refolded by MLFTPP showed an emission maximum at 342 nm (Fig. S3A), which is characteristic of the properly folded structure [13]. The CD spectrum of MLFTPP-refolded malETrx was similar to that of WT-thioredoxin showing a folded native-like secondary structure (Fig. S3B). ANS did not bind to either WT-thioredoxin or MLFTPP-refolded malETrx (Fig. S3C). Table S1 of the supplementary material shows the results of insulin aggregation assay for thioredoxin, which confirmed that the MLFTPP-refolded malETrx was active and comparable to WT-thioredoxin in its biological activity.

The melting temperatures (T\textsubscript{m} values) of the refolded proteins were similar to the T\textsubscript{m} values of the native WT proteins (Table 2).

Refolding of MBP mutants (224D and 264D) from inclusion bodies

MBP is a monomeric protein with two domains. It consists of 370 amino acid residues. It is involved in maltose uptake and che-

<table>
<thead>
<tr>
<th>Protein</th>
<th>Smart polymer and its concentration used (%) (w/v)</th>
<th>Ammonium sulfate (%) (w/v)</th>
<th>t-Butanol (v/v)</th>
<th>Buffer used</th>
</tr>
</thead>
<tbody>
<tr>
<td>CcdB-F17P</td>
<td>Eudragit L-100 (0.5)</td>
<td>30</td>
<td>1:2</td>
<td>50 mM Tris–HCl (pH 7.5)</td>
</tr>
<tr>
<td>CcdB-M97K</td>
<td>Eudragit L-100 (0.5)</td>
<td>30</td>
<td>1:2</td>
<td>50 mM Tris–HCl (pH 7.5)</td>
</tr>
<tr>
<td>CD4D12</td>
<td>Eudragit L-100 (0.5)</td>
<td>30</td>
<td>1:2</td>
<td>50 mM Tris–HCl (pH 7.5)</td>
</tr>
<tr>
<td>malETrx</td>
<td>Eudragit L-100 (0.5)</td>
<td>30</td>
<td>1:2</td>
<td>50 mM Tris–HCl (pH 7.5)</td>
</tr>
<tr>
<td>MBP224D</td>
<td>Cationic starch (0.3)</td>
<td>45</td>
<td>1:1</td>
<td>50 mM Tris–HCl (pH 7.5)</td>
</tr>
<tr>
<td>MBP264D</td>
<td>Cationic starch (0.3)</td>
<td>45</td>
<td>1:1</td>
<td>50 mM Tris–HCl (pH 7.5)</td>
</tr>
<tr>
<td>ScFv b12</td>
<td>Protanal alginate (1.0)</td>
<td>30</td>
<td>1:2</td>
<td>50 mM Tris–HCl (pH 7.0)</td>
</tr>
<tr>
<td>ScFab b12</td>
<td>Protanal alginate (1.0)</td>
<td>30</td>
<td>1:2</td>
<td>50 mM Tris–HCl (pH 7.0)</td>
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Note. After mixing in appropriate amounts of all the ingredients, the mixture was centrifuged at 3000g for 5 min for the formation of three phases. The temperature used during the MLFTPP process was 25 °C in all of the cases.
The two mutants 224D and 264D involved mutations of residues methionine to aspartate and alanine to aspartate, respectively, which are buried in the wild type. Both mutants formed inclusion bodies when expressed in *E. coli* BL21(DE3) [36].

A few years ago, we reported that soluble MBP and its fusion proteins can be purified using AP by binding to cationic starch [21]. Inclusion bodies of both MBP mutants were refolded by MLFTPP using cationic starch as shown by fluorescence-based maltose binding activity assay of the refolded proteins (see Fig. S4A of the supplementary material). The ANS did not bind to WT-MBP or any of the MLFTPP-refolded MBP mutants (Fig. S4B).

**Refolding of ScFv b12 and ScFab b12 from inclusion bodies**

The antibody fragment ScFv b12 consists of 254 amino acids with an isoelectric point of approximately 9.1, whereas ScFab has 479 amino acids with an isoelectric point of approximately 8.8 [13]. Both antibody fragments neutralize HIV-1 [42]. Both ScFv b12 and ScFab b12 were refolded from their inclusion bodies by MLFTPP using Protanal LF as a smart polymer (macro-affinity ligand), as shown by intrinsic fluorescence emission and CD spectra (see Fig. S5 of supplementary material). Both refolded antibody fragments were assayed for their biological activity of binding to HIV-1 gp120. The binding of ScFv b12 and ScFab b12 to surface-immobilized full-length gp120 was determined in vitro using SPR.
The $K_D$ values calculated for ScFv b12 (242 nM) and ScFab b12 (210 nM) are quite similar, as expected given that they have identical residues in the gp120 binding site (Table 3). The ScFv b12 has nearly 3-fold higher $k_{on}$ and $k_{off}$ rates compared with ScFab b12, likely because of its smaller size. However, the overall binding constants remain approximately the same.

We had earlier mentioned that MLFTPP has greater potential for simultaneous purification of proteins. In the case of ScFv b12, refolding was also reported without extensive washing of inclusion bodies before dissolving them in the urea. These washing steps are known to be necessary for removal of adhering impurities in the inclusion bodies [4,9]. MLFTPP starting with unwashed inclusion bodies of ScFv b12 was still found to give the refolded protein with the same yield and purity (on SDS–PAGE analysis) (see Fig. S6 of supplementary material), as obtained by MLFTPP carried out with extensively washed inclusion bodies prior to the solubilization step (Fig. 4B and Table 4).

### Purity and yields of the refolded proteins

All proteins refolded by MLFTPP were also simultaneously purified, as shown by SDS–PAGE analysis (Fig. 4).

Table 4 shows the refolding yields obtained with MLFTPP and AP for various proteins. The yields with MLFTPP were better for CcdB-F17P, CcdB-M97K, CD4D12, malETrx, and ScFab b12, whereas comparable yields to AP or TPP were obtained in other cases [13] or TPP [9]. It may be noted that many of the proteins refolded by MLFTPP (e.g., CcdB mutants, malETrx, MBP mutants) have been found to be aggregation prone and could not be refolded by either dilution or chromatographic methods [9].
Table 3
SPR-determined kinetic parameters for binding of ScFv b12 and ScFab b12 to surface-immobilized full-length gp120.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScFv b12</td>
<td>$7.2 \times 10^3$</td>
<td>$1.8 \times 10^{-3}$</td>
<td>242</td>
</tr>
<tr>
<td>ScFab b12</td>
<td>$2.1 \times 10^3$</td>
<td>$4.5 \times 10^{-4}$</td>
<td>210</td>
</tr>
</tbody>
</table>

Table 4
Comparison of refolding yields* (mg L$^{-1}$ culture) obtained by MLFTPP and affinity precipitation.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Protein</th>
<th>Refolding yields obtained by MLFTPP (mg L$^{-1}$ culture)</th>
<th>Refolding yields obtained by affinity precipitation (mg L$^{-1}$ culture)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CcdB-F17P</td>
<td>$40 \pm 2$</td>
<td>$32 \pm 2$</td>
</tr>
<tr>
<td>2</td>
<td>CcdB-M97K</td>
<td>$38 \pm 2$</td>
<td>$30 \pm 2$</td>
</tr>
<tr>
<td>3</td>
<td>CD4D12</td>
<td>$10 \pm 1$</td>
<td>$8 \pm 1$</td>
</tr>
<tr>
<td>4</td>
<td>maeETrx</td>
<td>$62 \pm 2$</td>
<td>$58 \pm 2$</td>
</tr>
<tr>
<td>5</td>
<td>MBP224D</td>
<td>$40 \pm 2$</td>
<td>$40 \pm 2$</td>
</tr>
<tr>
<td>6</td>
<td>MBP264D</td>
<td>$42 \pm 2$</td>
<td>$43 \pm 2$</td>
</tr>
<tr>
<td>7</td>
<td>ScFv b12</td>
<td>$30 \pm 2$</td>
<td>$30 \pm 2$</td>
</tr>
<tr>
<td>8</td>
<td>ScFab b12</td>
<td>$28 \pm 2$</td>
<td>$26 \pm 2$</td>
</tr>
</tbody>
</table>

Note: In all of the cases, MLFTPP required approximately 10 min. This included approximately 5 min of centrifugation time. Low-speed centrifugation (3000g) facilitated separation of phases. In comparison, the affinity precipitation method [13] required approximately 60 min.

* Method for calculating yield (mg L$^{-1}$ culture): [(total purified protein (mg) × fraction of pure protein)/culture volume (ml)] / 1000.

References


