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Rapid and Efficient Purification of RNA-Binding Proteins: Application to HIV-1 Rev

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Abstract

Non-specifically bound nucleic acid contaminants are an unwanted feature of recombinant RNA-binding proteins purified from *Escherichia coli* (*E. coli*). Removal of these contaminants represents an important step for the proteins' application in several biological assays and structural studies. The method described in this paper is a one-step protocol which is effective at removing tightly bound nucleic acids from over-expressed tagged HIV-1 Rev in *E. coli*. We combined affinity chromatography under denaturing conditions with subsequent on-column refolding, to prevent self-association of Rev while removing the nucleic acid contaminants from the end product. We compare this purification method with an established, multi-step protocol involving precipitation with polyethyleneimine (PEI). As our tailored protocol requires only one step to simultaneously purify tagged proteins and eliminate bound cellular RNA and DNA, it represents a substantial advantage in time, effort, and expense.

Keywords

HIV-1; Rev; RRE; urea denaturation; immobilized metal affinity chromatography; on-column refolding; PEI

Introduction

RNA, in its varied forms, interacts with proteins to carry out fundamental roles in the cell. The understanding of molecular recognition events, including those involved in assembly of macromolecular complexes consisting of both protein and RNA, are a particular challenge for *in vitro* biochemical, biophysical, and structural study. Purification of an overexpressed protein is essential for the subsequent detailed characterization of the protein and its complexes. For this purpose, purity and maintaining the native conformation of the protein are the most important criteria. RNA-binding proteins are known to have a relatively broad specificity in binding to RNA; thus, such proteins may bind to host RNA or DNA during overexpression and co-purify during the purification process. Conventional purification steps to remove nucleic acid contaminants include nuclease digestion, chemical treatments, or ultracentrifugation steps [1]. Additionally, the precipitation of nucleic acids with polyelectrolytes has been proposed and tested for *Escherichia coli* (*E. coli*) [2,3] and wheat

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germ [4]. Among polyelectrolytes, polyethylenimine (PEI) has been widely employed [5–7]. PEI is a positively charged polyelectrolyte (pK_a 9.7) with the structural formula $(-CH_2-NH-CH_2-)_n$ [8]. PEI binds negatively charged DNA and RNA, and the resulting precipitates can be removed by centrifugation. Although extensively used, PEI treatment of cellular extract or protein solution is inconvenient. The amount necessary to selectively precipitate non-target proteins and nucleic acids must be determined empirically. The binding of PEI to nucleic acids strongly depends on the ionic strength of the solution. Frequently, the target protein is found in the precipitate and additional purification steps are required to recover the protein of interest. In addition to cost and toxicity, PEI interferes strongly in all standards of protein estimation and prevents the accurate quantification of the protein concentration during purification [9].

We describe an alternative route for the removal of contaminant nucleic acids utilizing chemical denaturation and successive on-column refolding. In particular, we report RNA contamination of the recombinant HIV-1 Rev protein and propose a fast and reliable way to resolve the contamination problem yielding large amounts of native, functional protein. HIV-1 replication critically depends on Rev, which is encoded by a multiply spliced message [10, 11]. Rev functions as a sequence-specific RNA binding protein that activates the nuclear export of intron-containing viral mRNA transcripts to the cytoplasm. A plethora of studies have highlighted the unequivocal importance of oligomeric binding of Rev to the Rev Response Element (RRE) in nuclear export of viral RNA transcripts. Only oligomeric Rev-RRE complexes can interact with the CRM1 (or exportin 1) cellular export factor resulting in Rev-dependent export of full length and partially spliced mRNAs [12–14].

The tight association of RNA-binding proteins such as Rev with contaminating cellular nucleic acid is detrimental to a variety of assays, including RNA-binding analysis, a variety of spectroscopic studies, and structural analysis. Here, we describe a fast and simple one-step affinity-purification method for the isolation of specific RNA-binding proteins. Our protocol is based on chemical denaturation and subsequent on-column renaturation of a hexahistidine (His_6)-tagged HIV-1 Rev protein. Large amounts of soluble protein with purity greater than 95% and free of contaminating nucleic acid can routinely be obtained. This purified (His_6)-tagged Rev is homogeneous as judged by its secondary structure content and highly active in gel-mobility RRE stem-loop II binding assays. The adaptable protocol can be a versatile tool for the isolation of unknown RNA-binding proteins.

Material and Methods

Bacterial expression of (His_6)-tagged recombinant Rev

Plasmid encoding (His_6)-tagged Rev protein was transformed into *E. coli* strain BL21(DE3). Transformed cells were grown at 37 °C in one liter of LB media (containing 100 μ M/ml ampicillin) to OD_{600} of approximately 0.5. Protein expression was induced by addition of 1 mM isopropylthio- β -D-galactoside (IPTG). After addition of IPTG, the cells were incubated 4 h at 37 °C. The cells were harvested by centrifugation and stored at –80 °C. Wet cell pellets weighted approximately 3 g per 1 liter of LB medium.

Purification of (His_6)-tagged recombinant Rev using PEI (protocol A)

Frozen pellets were thawed and resuspended in lysis buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 25 mM imidazole) and lysed by sonication on ice. Insoluble cell debris was removed from the cell lysate by centrifugation at 4 °C for 20 minutes (15000g); subsequently, the cleared lysate was filtered through a 0.45 μ m filter. The filtrate was purified by immobilized metal affinity chromatography (IMAC) on a 1 ml Sepharose HisTrap FF nickel column (GE Healthcare). The protein was eluted with a linear gradient from the His-binding buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium

chloride, 1 mM DTT, 0.02% sodium azide, 25 mM imidazole) to the His-elution buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 500 mM imidazole) at 1 ml/min over 20 minutes. The fractions containing the protein of interest were collected, pooled, and the ionic strength of the solution was increased to 1 M sodium chloride. All successive steps were performed at 4 °C or on ice. PEI was added to the final concentration of 0.5% (w/v) and the solution was incubated for 1 h. The resulting suspension was centrifuged at 15000g for 20 min to remove PEI-nucleic acid complexes. The protein was recovered from the excess of PEI present in the supernatant by precipitation with 75% ammonium sulfate. After overnight incubation, this suspension was centrifuged, and the pellet containing the protein was re-suspended in binding buffer (50 mM sodium phosphate, pH 7.4, 200 mM sodium chloride, 1 mM DTT, 0.02% sodium azide), applied onto a 1 ml HiTrap SP XL column, and eluted with a linear gradient into elution buffer (50 mM sodium phosphate, 2 M sodium chloride, 1 mM DTT, 0.02% sodium azide, pH 7.4). Protein samples were concentrated 8-fold using an Amicon Ultra-15 (Millipore) with a 5 kDa MWCO membrane. Residual traces of imidazole were removed by dialyzing the eluate at 4 °C overnight against one liter of storage buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide) containing 10% glycerol. Aliquots were stored at -80 °C for further characterization. The concentration of proteins contaminated with nucleic acids was determined using a Bradford protein assay (BioRad) while concentrations for the pure protein were determined by measuring the absorbance at 280 nm and an extinction coefficient (ϵ) of $8600 \text{ cm}^{-1} \text{ M}^{-1}$, as determined by amino acid sequence data [15]. The purity of Rev was monitored after each purification step by SDS-PAGE using 4–20% gradient gels (BioRad).

Purification of (His₆)-tagged recombinant Rev using urea denaturation/on-column refolding (protocol B)

Frozen pellets were thawed and resuspended in His-binding buffer containing 8 M urea (8 M urea, 50 mM sodium phosphate, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 25 mM imidazole, pH 7.4) and lysed by sonication on ice. Insoluble cell debris was removed from the cell lysate by centrifugation at 4 °C for 20 minutes (15000g); subsequently, the cleared lysate was filtered through a 0.45 μm filter. The urea-containing filtrate was purified by IMAC on a pre-equilibrated 1 ml Sepharose HisTrap FF nickel column (GE Healthcare) applied at a flow-rate of 1 ml/min rate. On-column (His₆)-tagged Rev renaturation was achieved at a reduced flow rate of 0.5 ml/min with a linear gradient from 8 to 0 M urea over thirty column volumes. The renatured protein was eluted from the column with the His-elution buffer (50 mM sodium phosphate, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide, 500 mM imidazole, pH 7.4) at 1 ml/min over 20 minutes. Protein samples were concentrated 15-fold using an Amicon Ultra-15 (Millipore) with a 5 kDa MWCO membrane. Residual traces of imidazole were removed by dialyzing the eluate at 4 °C overnight against one liter of storage buffer (50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, 0.02% sodium azide) containing 10% glycerol. Aliquots were stored at -80 °C prior to further characterization. The successful renaturation of proteins by gradually reducing the amount of urea potentially presents a serious obstacle for other target proteins. Alternatively, lower, nondenaturing concentrations of urea combined with higher ionic strength can be investigated, e.g. 1 M urea in combination with 1 M NaCl (see Results).

Circular dichroism measurements

Circular dichroism (CD) measurements of purified (His₆)-tagged Rev were performed on a temperature-controlled AVIV spectropolarimeter. CD spectra were recorded at 5 °C between 198 and 260 nm, with a 0.1 cm path length cell, a wavelength increment of 1 nm, an averaging time of 5 seconds, and an equilibration time of 5 minutes. The baseline was corrected by subtracting the spectra of the respective buffers collected under identical conditions. Spectra from three scans were averaged. (His₆)-tagged Rev samples at 10 μM concentration in the

storage buffer were centrifuged at 5000g for 10 min at 5 °C prior to further analysis. The concentration of the supernatant containing the recombinant Rev protein was assessed by measuring the absorbance at 280 nm. Deconvolution of the CD spectrum was performed using the CDPPro suite software package consisting of the CONTIN/LL, CDSSTR, and SELCON3 software packages [16,17]. The chosen IBasis 4 parameter [18] contains a large reference set of 43 soluble proteins. The reported overall secondary structure percentages represent averaged values derived from all three programs.

Urea-induced denaturation

Urea-induced denaturation of (His₆)-tagged Rev was monitored by CD in the wavelength range of 210–260 nm at 25 °C. Rev solutions at 10 μM concentration were mixed with varying amounts of stock solution containing 10 M urea. The buffer in all denaturation reaction was 50 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 1 mM DTT, and 0.02% sodium azide. Unfolding was monitored in the range of 0 to 8 M urea. Spectra represent the average of three scans for each urea concentration. The urea-unfolding profile of (His₆)-tagged Rev is described by the change of the molar ellipticity value at 222 nm, indicative of an α-helical secondary structure, as a function of denaturant concentration.

Chemical denaturation data were analyzed by direct non-linear least-squares fitting of the observed CD signal (Y) to a two-state model of a single unfolding transition between folded (F) and unfolded (U) states [19]:

$$Y = \alpha_i ([\theta]_F - [\theta]_U) + [\theta]_U \quad (1)$$

where $[\theta]_F$ is the ellipticity at which the molecule is fully folded and $[\theta]_U$ is the ellipticity of the fully unfolded molecule. The fractional population of the unfolded form (α_i) is determined from the equilibrium constant for folding:

$$K_{Fi} = \exp(-\Delta G_i / RT) \quad (2)$$

where R is the gas constant, which equals 1.98 cal/mol, and T is the absolute temperature. ΔG_i is calculated using the linear extrapolation model (LEM) [20]:

$$\Delta G_i = \Delta G^0 + m[D] \quad (3)$$

where ΔG^0 is the standard free energy of unfolding in the absence of denaturant and m is the slope which characterize the change in ΔG_i with $[D]$. The denaturant concentration at the midpoint of the transition, $[D]_{1/2}$, is equal to $-\Delta G^0/m$.

Electrophoretic mobility shift assay

RRE Stem-loop II, a 70 nucleotides RNA, was synthesized by run-off transcription using T7-polymerase and pUC19-RRE-Stem-loop II linearized with SmaI (1X transcription buffer: 24 mM NTPs, 42 mM MgCl₂, 200 mg/ml T7-polymerase, 100 U/ml RNaseOUT (Invitrogen), and 75 mg/ml template DNA). Synthesized RNA was purified by reverse phase ion-pairing chromatography (RP-IP; Phenomenex Oligo-RP 10 × 250 mm column; buffer A: 50 mM TEAA, 5% acetonitrile, pH 7.5; buffer B: 100% acetonitrile). RRE Stem-loop II RNA eluted at approximately 11% buffer B. Fractions containing purified RRE Stem-loop II RNA, as determined by PAGE analysis, were combined and concentrated/desalted in a 15 ml Centrplus YM-3 concentrator (Millipore) against RNase-free Milli-Q water.

The purified Stem-loop II RNA (100 μg) was dephosphorylated with 50 U of antarctic phosphatase (New England Biolabs, Inc.) for 2 h at 37°C in a 50 μl reaction. To separate dephosphorylated RNA (shortest retention time) from RNA still retaining 5' phosphate groups (longest retention time), phosphatase treated RNA was re-fractionated by RP-IP as described above. Dephosphorylated RNA was combined, concentrated, and precipitated with 2.5

volumes of 100% ethanol and 0.1 volume 3 M sodium acetate, pH 5.2. Stem-loop II RNA was 5'-labeled with [γ - 32 P] ATP in a 100 μ l reaction at 37°C (14 μ g RNA, 5 μ l of [γ - 32 P] ATP 6000 Ci/mmol, 50 U T4 polynucleotide kinase (Promega), 4 U RNaseOUT (Invitrogen)). Solid urea was added to a final concentration of 8 M to arrest the reaction. One hundred μ l of RNA loading buffer containing 8 M urea was added and labeled products separated in a 12.5% polyacrylamide gel (19:1) containing 8 M urea. Labeled Stem-loop II RNA was visualized by exposing the gel to film (Kodak Biomax AR). The radioactive band corresponding to full length Stem-loop II RNA was excised from the gel. RNA was extract from the gel matrix by passive diffusion in 5 volumes of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS) overnight. A second elution was performed for 3 h and both fractions combined and ethanol precipitated as described above. Radiolabeled-RNA was heated to 95°C and snap-cooled on ice. Afterwards, the radiolabeled-RNA was adjusted to the final concentration of 2 nM in 1 X QT buffer (10 mM HEPES, pH 7.5, 150 mM KCl, 5 mM DTT, 10% glycerol, 100 μ g/ml BSA, 100 μ g/ml yeast tRNA).

Stocks of Rev protein were diluted to 12 nM with 1X QT buffer. Binding reactions were performed by mixing equal volumes of RNA and protein in 96-well microtiter plates at the specified concentrations indicated. RNA and protein samples were incubated for 2 h at room temperature. Polyacrylamide gels (37.5:1) prepared with 0.5X TBE buffer (0.45 M Tris base, 0.45 M boric acid, 0.01 M EDTA, pH 8.3) were pre-heated for 1 h at 250 V. Samples were loaded and gels ran at 500 V for 1 h and 15 min in a water-cooled electrophoresis apparatus. Finally, gels were dried on Whatman paper (Schleicher & Schuell), and signals detected and quantified using a Storm 860 Phosphorimager (GE Healthcare). The counts in the second band from the bottom were considered the monomeric Rev-bound species while the counts in the third band from the bottom were considered the dimeric Rev-bound species. The data were fit using non-linear least squares analysis using Igor software (Wavemetrics). Apparent dissociation constants K_{D1} and K_{D2} , were calculated by simultaneous solution of the following equations for a three state model of stepwise binding [21–23]:

$$\begin{aligned} R_f([Rev_f]) &= 1/P \\ R_1([Rev_f]) &= K_1[Rev_f]/P \\ R_2([Rev_f]) &= K_1K_2[Rev_f]^2/P \end{aligned} \quad (4)$$

where $R_f([Rev_f])$ is the fraction of unbound stem II RRE while $R_1([Rev_f])$ and $R_2([Rev_f])$ represent the fractions of monomer and dimer Rev-bound states, respectively. $[Rev_f]$ is the concentration of RRE free Rev and is assumed to equal the total Rev concentration because radiolabeled RRE was added in trace. K_1 and K_2 are the binding constants:

$$\begin{aligned} K_1 &= [RRE \bullet Rev]/([RRE_f][Rev_f]) \\ K_2 &= [RRE \bullet Rev_2]/([RRE \bullet Rev][Rev_f]) \end{aligned} \quad (5)$$

where $[RRE \bullet Rev]$ and $[RRE \bullet Rev_2]$ are the concentrations of monomeric and dimeric Rev_n-RRE complexes, respectively. Finally P is the binding polynomial:

$$P = 1 + K_1[Rev_f] + K_1K_2[Rev_f]^2 \quad (6)$$

The binding constants were converted to dissociation constants by taking their inverse (i.e. $K_{D1} = 1/K_1$) within the fitting algorithm.

Results

Comparison of (His₆)-tagged Rev Purification Protocols

A recombinant gene encoding (His₆)-tagged Rev was expressed in *E. coli* at high levels (Fig 1, lane 2). After sonication of the cell lysate, the recombinant Rev protein is found primarily in the soluble fraction of *E. coli* lysate (Fig 1, lane 3). SDS-PAGE reveals that (His₆)-tagged

Rev protein was determined to have an apparent molecular weight of 20 kDa (Fig 2). The observed anomalous behavior on SDS-PAGE (the nominal molecular weight is 15 kDa) is consistent with that reported for other proteins rich in basic residues [24]. Following Protocol A (Fig 2 A), described in the Materials and Methods section, the cleared lysate was purified by nickel affinity chromatography (Fig 1, lane 4). Although the protein was determined to be 95% pure by Coomassie staining, the UV spectrophotometric analysis of the sample indicated a significant contamination with nucleic acids (Table 1). Subsequently, the ratio of absorbance at 260 nm vs. 280 nm was used to quantify nucleic content because of its high sensitivity to assess the purity of the Rev protein with respect to nucleic acid contamination [25–28]. Using common extinction values (for 1 mg/ml solutions free of Phenol and other extraneous contaminants) of 1.00 and 0.57 at 280 and 260 nm, respectively, for proteins and 10 and 20 at 280 and 260 nm, respectively, for nucleic acids, Glasel [25] derived an empirical equation (see Table 1) to estimate the nucleic acid contamination of isolated proteins with average aromatic residue composition. Accordingly, the measured ratio of 1.98 indicated that approximately 88% of the nickel affinity purified Rev protein was bound to endogenous nucleic acid from *E. coli*. Treatment of the purified (His₆)-tagged Rev with DNase and RNase identified the contaminants as primarily RNA (Fig S1) which is consistent with previous reports [29]. Contaminating RNA was removed by PEI precipitation [5]. This step was performed at high ionic strength (1 M NaCl) to reduce Rev's affinity for cellular nucleic acids. The PEI precipitate was removed by centrifugation and the pellet resuspended in an ammonium sulfate buffer to elute the target protein. PEI precipitation and ammonium sulfate fractionation were successful in removing nucleic acid contaminants as the supernatant containing the (His₆)-tagged Rev showed a 260:280 ratio of 0.64 (Table 1). The supernatant was then purified to homogeneity by a cation exchange column in order to eliminate the residual PEI and other minor contaminants. At this point, the sample exhibited a 260:280 ratio of 0.61 commonly associated with a protein preparation free of nucleic acid contaminants (Table 1). The absence of nucleic acid was corroborated by denaturing gel analysis (Fig S1). The target protein was judged sufficiently pure for RNA-binding assays and protein characterization by CD spectroscopy. However, purification of large amounts of (His₆)-tagged Rev using protocol A was difficult due to low yield (approximately 1 mg/L of media).

In light of the poor yield and the time consuming protocol previously described, we devised a simple and efficient purification method that simultaneously purifies recombinant Rev protein from other *E. coli* proteins and removes contaminating ribonucleic acids (protocol B) (Fig. 2 B). This protocol consists of: 1) denaturation of protein extracts with varying amounts of urea in presence of high salt concentrations, 2) capture of (His₆)-tagged Rev by nickel affinity chromatography, and 3) successive on-column renaturation (Fig 1B). It should be noted that in favorable case, complete denaturation of the target protein may not even be required. We experimentally determined the amount of urea required to achieve complete denaturation of the (His₆)-tagged Rev protein by CD (Fig 3). The urea-induced unfolding of Rev was monitored by the decrease in intensity of the relative band at 222 nm which indicates the loss of a α -helical conformation. Chemical denaturation was characterized by a single transition with a midpoint of 2 M. In order to analyze the effect of urea-induced denaturation, the cell pellets were re-suspended in a denaturing buffer containing 8 M urea as well as nondenaturing buffer containing only 1 M urea. The cell lysates were centrifuged after extensive sonication and independently applied to a nickel column. High and low denaturant concentrations were gradually reduced to zero and the protein eluted with increasing concentrations of imidazole. The purity of the protein fully denatured in 8 M urea was determined by SDS-PAGE and found to be greater than 95% (Fig 1, lane 8). In marked contrast to purification under native condition, the purified (His₆)-tagged Rev show a low 260:280 ratio (0.58) demonstrating the complete removal of nucleic acids (Table 1 and Fig S1). At this point, the recombinant Rev protein was considered sufficiently pure for further biophysical experiments. The relative yield of pure, renatured (His₆)-tagged Rev increased approximately ten-fold (9 mg/L of media) over that

obtained by the purification under native conditions. The purification of (His₆)-tagged Rev under nondenaturing conditions (1 M urea) was investigated in combination with a higher concentration (1 M) of sodium chloride to further reduce nonspecific interactions. This variant of our protocol B yielded a protein isolate which is virtually indistinguishable from the renatured (His₆)-tagged Rev in terms of yield (13 mg/L of media), purity (260:280 ratio of 0.75) and secondary structure content (see Supplementary Fig. S2).

CD analysis of (His₆)-tagged Rev following PEI precipitation or chemical denaturation

To examine potential conformational disparities between Rev proteins prepared according to the two different protocols A and B described above, we measured CD spectra. CD spectra of both Rev protein preparations are shown in Figure 4. Both spectra revealed considerable structure with two minima at 208 and 222 nm, characteristic of α -helical structure. The ratio of those minima, $[\theta]_{222:208}$, has been used as a criterion in several proteins to evaluate the presence of coiled-coil helices. For a non-interacting α -helix, the ratio has been shown to be 0.83 [30] while for two-stranded coiled-coils, the ratio was calculated to be 1.03. (His₆)-tagged Rev prepared according to protocols A and B exhibited a $[\theta]_{222:208}$ below 1 (0.88), characteristic of a protein containing a single-stranded α -helix [30–32] at 10 μ M. The quantitative analysis of both CD spectra indicated that both purification protocols produced a protein with very similar secondary structure composition (~ 38% α -helix, ~ 12% β -sheet, and ~ 50% unordered structures and turns) (Fig 4 C and D).

RNA-binding activity of (His₆)-tagged Rev protein following PEI precipitation or chemical denaturation

To assess the activity of (His₆)-tagged Rev, binding of Rev to an RNA transcript containing the RRE stem II sequence was studied by an *in vitro* electrophoretic mobility shift assay (EMSA). The RNA used is a 70-nucleotide truncated version of the RRE comprising the high affinity site (stem-loop IIB) and sufficient flanking regions (stem-loop IIC and stem IIA) to bind a total of two Rev monomers. (His₆)-tagged Rev purified using PEI precipitation (protocol A) or urea-denaturation followed by on-column refolding (protocol B) was incubated with radiolabeled RNA to form a specific RNA-protein complex over a range of protein concentrations. The addition of Rev protein prepared by either purification protocol decreased the electrophoretic gel mobility of the Stem II RRE fragment (Fig 5, lane b-l). The observed multiple discrete band shifts were dependent on the concentration of the purified protein reflecting both, specific Rev-RRE interaction and ability of Rev to oligomerize. Decreased gel mobility was not observed in the absence of protein (Fig 5, lane a). The quantitative analysis of this gel mobility shift assay allows for the calculation of the dissociation constants (K_{D1}) associated with the binding of one (K_{D1}) or two (K_{D2}) Rev monomers. Here, we assumed that the second Rev copy binds to the previously formed 1:1 Rev:RRE involving the high-affinity binding site stem-loop IIB [23]. The second binding event is facilitated by both, protein-protein and secondary protein-RNA interaction. The apparent equilibrium dissociation constants for direct titration electrophoretic mobility shift were determined from a fit of the data using nonlinear least squares regression (see Materials and Methods). The estimates of K_{D1} and K_{D2} were 105 ($\pm$ 32) and 224 ($\pm$ 112) nM for Rev purified according to PEI precipitation (protocol A) and 141 ($\pm$ 60) and 179 ($\pm$ 113) nM for Rev purified according to urea-denaturation followed by on-column refolding (protocol B), respectively. These EMSA results indicate that (His₆)-Rev proteins purified according to protocol A or B are indistinguishable and both capable of binding to stem II RRE with high affinity and to form oligomeric complexes.

Discussion

RNA-binding proteins play key roles in many cellular and viral processes. The essential regulatory HIV-1 protein Rev constitutes a paradigm for proteins that interact with RNA

through an arginine-rich motif. Consequently, Rev that is overexpressed in *E. coli* at high levels binds endogenous RNAs with high avidity. In particular, due to its RNA-binding domain (residues 33–55) featuring a positively charged stretch of arginines, RNA contaminations are an inherent problem of HIV-1 Rev purification schemes; an obstacle shared among many RNA-binding and positively charged proteins. Non-specific RNA binding of Rev has been demonstrated in crude cell extract [10] as well as purified protein [11,33–35]. The purification of HIV-1 Rev to near homogeneity has been described previously. The recombinant (His₆)-tagged HIV-1 Rev construct under investigation is comprised of 132 residues (MW of 15 kDa) and has a basic pI of 9.75. Contaminating nucleic acids reportedly were removed by a variety of approaches such as: ethanol precipitation from crude cell extracts [29,34,36] combinations of carboxymethyl-Sepharose with gel filtration chromatography [11], heparin-Sepharose affinity chromatography [37], purification in the presence of 6 M urea and subsequent stepwise renaturation [38], and precipitation with the polycation PEI [39]. Cochrane and co-workers [40] reported a purification of (His₆)-tagged Rev by IMAC under denaturing condition. However, the acidic condition (pH 2–5) chosen when eluting the protein appeared to compromise the proper refolding of Rev and contributed to unwanted aggregation of the protein. As a result, Rev showed low level of activity when “scrape-loaded” into cells.

We attempted to devise a protocol allowing us to overexpress and purify homogenous (His₆)-tagged Rev protein in a fast and reproducible manner. We compared the newly developed purification protocol that involves urea-denaturation with an established procedure based on PEI precipitation. Here, we employed polycationic PEI treatment to remove RNA contaminants from nickel column purified (His₆)-tagged Rev rather than treating crude extract [33,39]. The advantage of the nucleic acid precipitation in a purified protein solution is the reduced viscosity of the solution and the absence of other proteins that can compete with nucleic acids for the PEI binding. Although the PEI treatment was successful, we failed in our efforts to economically produce large amounts of (His₆)-tagged Rev protein required for structural and other biophysical studies. Moreover, we and others have pursued approaches that use recombinant techniques and selectively designed protein mutants to determine functionally important residues within Rev. This strategy inevitably requires not only the overexpression of many protein variants but also the purification of mutant proteins with e.g. impaired oligomerization properties [21,41,42].

A major concern with PEI precipitation is the requirement of empirical adjustments of PEI concentrations for each different protein construct and protein preparation. Varying protein concentrations as well as the physical characteristics of the protein of interest can interfere with PEI precipitation. Furthermore, since the preparation of consistent cell extracts is inherently difficult due to cell growth variables, an effective PEI precipitation is difficult to reproduce. The same reasoning applies to the successive ammonium sulfate precipitation.

The combination of the two precipitation steps compounds the complexity, because the PEI precipitation affects the effectiveness of the ammonium sulfate precipitation. These problems ultimately made PEI precipitations with successive ammonium sulfate fractionation unpredictable and unreliable without further, construct-specific optimization.

Thus, as an alternative route, we exploited a combination of chemical denaturation and successive on-column refolding in order to remove nucleic acids contaminants. Such on-column refolding procedures have been successfully employed to recover solubilized protein from inclusion bodies. Nickel chelating chromatography has been successfully applied to refold proteins [43] or enzymes [44] without compromising the native secondary structure or activity, respectively. We exploited an engineered hexahistidine affinity tag of Rev to perform on-column refolding using IMAC. The applied methodology is based on the reversible adsorption of the denatured Rev onto a solid support, thereby minimizing the effect of

differential oligomerization states of various Rev mutants, and subsequent gradual removal of denaturant to promote refolding. Urea-denatured Rev's binding capacity for RNA is negligible while the polyhistidine tag ensures protein immobilization onto the Ni-affinity matrix. The affinity tag ensures protein monomers to be spatially constrained during the refolding process, thus, preventing self-association upon gradual urea dilution. This gentle equilibrium refolding process is comparable to slow dialysis or dilution into a large volume of refolding buffer, with the exception that the protein is immobilized on a solid support rather than freely diffusing in solution. After renaturation, the adsorbed Rev molecules are recovered by increasing concentrations of imidazole in the elution buffer. It is often desirable to remove a (His₆)-tag after purification and our method is applicable to (His₆)-tagged Rev bearing a cleavage site between the tag and the encoded protein that is recognized by tobacco etch virus (TEV) protease (data not shown).

We subsequently evaluated and compared purity, yield, and answered the question whether the protein maintained its native conformation. Therefore, the proteins prepared according to purification protocols A and B were subjected to biophysical characterization to compare their conformational and specific RRE-binding properties. Proteins prepared were found to be functional as shown by its RNA binding ability monitored by EMSA. The secondary structure of the both protein preparations is nearly identical. The secondary structure composition is in agreement with previous CD studies on HIV-1 Rev [29,38,45,46].

In conclusion, our previous protocol for producing Rev from *E. coli* involved several steps with low overall yield and poor reproducibility when working with Rev protein variants: PEI and ammonium sulfate precipitation. The new protocol described here, which is much faster and reproducibly leads to a higher yield of native protein, involves a single IMAC purification step under denaturing conditions and successive on-column refolding. In addition, spectroscopic characterization by CD indicates that the recombinant protein adopts a folded homogeneous structure capable to specifically recognize its cognate RRE target with high affinity.

The devised protocol B is highly adaptable; results obtained with nondenaturing amounts of urea in combination with higher ionic strength suggest that the concentrations of denaturant and salt are adjustable parameters. A modified purification protocol using nondenaturing conditions represents an attractive alternative for target proteins that may not readily refolded after chemical denaturation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Comparison between the purification of (His₆)-tagged Rev using either (A) PEI or (B) urea denaturation protocols

Aliquots from individual steps of the purification of (His₆)-tagged Rev by the PEI or the urea protocols were subjected to electrophoresis on a 4–20% acrylamide gel. Lane 1, protein markers; lanes 2 and 6, total *E. coli* intracellular proteins; lanes 3 and 7, total soluble proteins; lanes 4 and 8, fractions (1 µg) purified by nickel column; lane 5, fraction (1 µg) purified from cationic exchange column. The molecular weight (kDa) of the marker proteins is indicated on the left.

Figure 2. Flowcharts of the purification protocols adopted to remove nucleic acid contaminants from (His₆)-tagged Rev protein

(A) Purification requires a multi-step protocol in order to remove contaminating nucleic acid. Following an initial purification by immobilized-metal affinity chromatography (IMAC), nucleic acids are effectively removed by PEI precipitation and ammonium sulfate fractionation. A final purification by a cationic exchange column yields pure (His₆)-tagged Rev without nucleic acid contamination. (B) Devised one-step purification of (His₆)-tagged Rev based on urea denaturation followed by successive on-column refolding. After urea denaturation, (His₆)-tagged Rev is refolded on IMAC ensuring the removal of contaminants in the final preparation.

Figure 3. Urea-induced unfolding transition of (His₆)-tagged Rev

The change of the dichroic signal at 222 nm (expressed as molar ellipticity per residue, θ) for (His₆)-tagged Rev at 10 μ M concentration was monitored at 25 °C as a function of increasing concentrations (from 0 to 8 M) of urea. Data was fitted using Eq. 1–3 (solid line).

Figure 4. CD analysis of purified (His₆)-tagged Rev

CD spectra of purified Rev following nucleic acid removal by (A) PEI precipitation or (B) urea denaturation/on-column refolding. Shown is the molar ellipticity per residue (θ) corrected for the background buffer. (C) and (D) Multicomponent analysis of the CD spectra of (His₆)-tagged Rev purified by (C) PEI precipitation or (D) urea denaturation/on-column refolding, respectively. The CD spectra A and B were deconvoluted using the algorithms CONTIN/LL (black bars), SELCON 3 (light gray bars), and CDSSTR (dark gray bars). Bars labeled “unordered” include estimated percentage of unordered structures and turns.

Figure 5. Gel-mobility shift assay of HIV-1 RRE stem II with (His₆)-tagged Rev protein
 All samples contained 1×10^{-9} M radiolabeled RNA; samples b-l contained 12.5, 25, 50, 100, 200, 400 800, 1000, 2000, 3000, 4000 $\times 10^{-9}$ M (His₆)-tagged Rev purified according the (A) PEI or (B) the urea denaturation/on-column refolding protocol, respectively. Samples were resolved on 8% (w/v) polyacrylamide gel cast run at room temperature in a water-cooled electrophoresis apparatus. Observable species are designated F (free RNA) or numbered (1–2) to indicate the (His₆)-tagged Rev:RNA ratio of the corresponding complexes.

Table 1

Protein yields and nucleic acid contents of HIV-1 (His₆)-tagged Rev purified by the PEI precipitation (A) or urea-denaturation/on-column refolding (B) protocols.

Method of removal of nucleic acids contaminants	Purification step	Total protein (mg/L of media)	OD ₂₆₀ :OD ₂₈₀	% nucleic acid ^c
(A) PEI precipitation	Nickel column	6 ^a	1.98	88
	PEI precipitation and ammonium sulfate fractionation	2.5 ^b	0.64	1
	Cationic exchange	1 ^b	0.61	0
(B) Urea denaturation/On-column refolding	Denaturing nickel column	9 ^b	0.58	0

^aProtein concentration estimated by Bradford assay.

^bProtein concentration estimated by the absorbance at 280 nm.

^cCalculated using the equation: $\%N = (11.16R - 6.32)/(2.16 - R)$ where $\%N$ = percent of nucleic acids, $R = OD_{260}:OD_{280}$, [26].