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High-throughput identification of refolding conditions for LXRβ without a functional assay

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Abstract

The expression of human genes in bacteria is often one of the most efficient systems for generating proteins for drug discovery efforts. However, expression of mammalian cDNAs in *Escherichia coli* often results in the production of protein that is insoluble and misfolded and thus requires the development of a successful refolding procedure to generate active protein. To accelerate the process of developing protein refolding protocols, we have developed a semi-automated screening and assay system that utilizes an incomplete factorial approach to sample a large "space" of refolding conditions based on parameters known to influence protein stability and solubility. Testing of these conditions is performed readily in a 96-well plate format with minimal sample manipulation. The folded protein is resolved and detected using an HPLC equipped with a mini-column and a highly sensitive fluorescence detector. This simple method requires only a small amount of protein for the entire screen (<1 mg), and most importantly, a functional assay is not required to assess the refolding yields. Here, we validate the utility of this screening system using two model proteins, IL13 and MMP13, and demonstrate its successful application to the refolding of our target protein, the ligand-binding domain of rat liver X receptor β.

Keywords: Refolding screen; Inclusion bodies; Protein-folding; Liver X receptor β

With the advances in molecular biology, laboratory automation, and computational science, a vast amount of genomic data from various organisms, including *Homo sapiens*, has now become available. The pharmaceutical industry is currently putting forth tremendous effort in the area of functional genomics and structural genomics in attempts to decipher functions and structures of protein encoded by genes, with the ultimate goal of identifying novel targets for drug discovery and development. The availability of these human proteins in large quantities will facilitate the drug discovery process in many ways, including assay development, high-throughput screening, lead characterization, and structure-based drug design.

The challenge of studying proteins on a genome-wide scale requires the development of high-throughput approaches in protein expression and purification. *Escherichia coli* remains the first choice among all hosts used for heterologous protein expression because it provides efficient and cost-effective system to produce recombinant proteins at high expression levels. Despite all advantages offered by the system, high-level expression of many recombinant proteins in *E. coli* often results in the formation of insoluble, inactive aggregates known as inclusion bodies. In such cases, it is necessary to develop a procedure to refold the protein into its native conformation [1–5]. The general strategy for protein renaturation involves inclusion body isolation; solubilization in high concentration of chaotropic reagents such as urea or guanidine–HCl (GdmHCl);[1] further chromatographic purification of the denatured proteins; and finally, refolding into the native state

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[1] *Abbreviations used:* Arg, L-arginine; CD, circular dichroism; DTT, dithiothreitol; GdmHCl, guanidine–hydrochloride; GSH/GSSG, reduced/oxidized glutathione; IB, inclusion body.

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via the controlled removal of excess denaturant. While the efficiency of the first few steps can be relatively high, with procedures applicable for almost all proteins expressed in inclusion bodies, identifying efficient renaturation conditions is usually the rate-limiting step. Specifically, gradual removal of the denaturant is often performed in a buffer containing a suitable redox system and of other folding promotion agents (the "refolding buffer"), the compositions of which are unique to each protein and are in most cases developed empirically. The buffer exchange steps are typically carried out by means of dilution, dialysis, diafiltration, or on-column refolding [6]. Dilution has the advantage of refolding at low protein concentrations (<10 μg/ml) thereby minimizing aggregation, but impractical at micro-liter scale when searching for a suitable refolding buffer because of the limited material available for further characterization. Refolding by dialysis and diafiltration at medium scale is amenable at higher protein concentrations (~500 μg/ml) but precipitation and aggregation can significantly reduce the refolding yields. On-column refolding has shown some promising results [6], although it might be protein specific and protein–matrix interactions can prevent refolding [7].

Ideally, the search for refolding conditions could be performed by exploiting a large number of buffer systems and accelerated by automation. Several screening systems for recombinant protein refolding have been reported in the literature. Gouaux's group designed a refolding screen, which includes 16 different conditions of 12 reagents that are known to favor the native state formation. A fractional factorial approach was used to examine a small, but representative subset of the full factorial. Several model proteins, including lysozyme, carbonic anhydrase B, and the ligand-binding domains (LBDs) from the glutamate and kainate receptors, were refolded successfully using this system [8,9]. Hampton Research (Laguna Niguel, CA) is currently marketing this screen as a protein refolding kit. Pierce Biotechnology (Rockford, IL) is also marketing a similar kit with nine different refolding conditions. Although these commercial refolding kits have been shown to be useful in rapidly screening refolding conditions for a number of proteins, one major limitation is the method of detecting the correctly folded material. The most common approach is a functional assay [10]. This is not an issue with well-characterized proteins but for other less well-studied proteins, the availability of a functional assay that is sensitive enough to detect small amounts (<1% yields) of refolded proteins may not be feasible. Other biophysical analyses such as a solubility test, dynamic light scattering, circular dichroism (CD), protease sensitivity [11], etc. have not been generally applicable to a large number of proteins (see review by Middleberg) [7] and are difficult to implement in a simple and high-throughput mode to analyze a large number of conditions.

We have developed a system that allows rapid screening of refolding conditions over a "large refolding space" without the need of a functional assay. Like the commercial refolding screens, our conditions were generated using an incomplete factorial approach to produce an unbiased, generic folding matrix to identify the best refolding conditions for a given protein target. A total of 81 conditions were identified based on factors that are known from the literature to promote folding and minimize aggregation in vitro. Both folding and dialysis steps were done in a 96-well plate format so that all 81 conditions were tested simultaneously. A combination of an initial dilution step followed by dialysis allows the refolding to occur at medium to low protein concentrations that are still sufficient for subsequent direct analysis. No further concentrating of the samples is necessary. An HPLC equipped with an autosampler, a fluorescence detector, and a micro-analytical column was used to separate folded protein from misfolded and aggregated species. No more than 10 μg of each sample was injected directly from the plate for analysis. This separation/detection method was based on our past observations, that in many cases, folded protein behaved very differently from its misfolded and aggregated counterparts on at least one chromatographic resin, such as ion-exchange, reverse phase, hydrophobic interaction (HIC), gel filtration, etc. In an analogy to this concept, an analytical HIC has been used in conjunction with tryptophan fluorescence spectroscopy or 1D ^{1}H NMR to evaluate refolding conditions [12,13]. In the case of ion-exchange chromatography, misfolded and aggregated material tends to elute much later or not at all in a linear NaCl gradient up to 1.0 M [6]. Despite the large number of conditions tested here, less than 1 mg of protein is required for the entire screen. Furthermore, detection by fluorescence provides a high degree of sensitivity so that folded protein can be detected in the conditions with less than 1% refolding yield. Because this miniaturized refolding screen does not rely on a functional assay to evaluate refolding efficiency, it eliminates the time needed to develop a sensitive assay and it can also be applied for those proteins where such an assay is not available. Here, we describe the design and application of our approach and present examples of its use on a number of protein targets. IL13 is a single domain, small cytokine (short chain 4-helix bundle), with two pairs of disulfide bonds. It has a molecular weight of 12.5 kDa. The protein was expressed in *E. coli* exclusively as inclusion bodies and its structure has been solved using the refolded material [14,15]. The second protein we tested was the procatalytic form of MMP13. It is a multidomain metalloprotease with a bound Zn^{2+} at the active site. It also requires Ca^{2+} for structural integrity. The zymogen form of MMP13 is expressed in *E. coli* as insoluble inclusion bodies and the protein can be refolded and activated in vitro [16,17]. MMP13 has no disulfide bridges and the molecular weight of the procatalytic form is 28.1 kDa. The third protein we have chosen is the LBD of the nuclear receptor Liver X Receptor β (LXRβ) [18,19]. LXRβ has no disulfide bonds and its LBD has a molecular weight of ~30 kDa. The X-ray structure shows that LXRβ forms a homodimer [20,21]. LXRβ-6×His was expressed in *E. coli* and the majority of the protein is in inclusion bodies.

Materials and methods

General refolding matrix

Over the years protein chemists have explored many different buffers, solvents, salts, and additives to increase the solubility and stability of proteins. This wealth of knowledge was applied to the refolding of many recombinant proteins as seen in the literature (see reviews [1–5]). We have gathered most of the literature from the past 20 years on protein refolding and evaluated and summarized many conditions and factors that are thought to affect refolding (Table 1). It would be ideal to test every combination of these factors in different concentrations and screen the entire "refolding space," which would include thousands of conditions at only one protein concentration. Obviously this would be an enormous task and cannot be easily accomplished in a short time. Instead we chose some major factors from each category (listed with superscript a in Table 1) as the starting points and used a design of experiment software DESIGN-EXPERT (Stat-Ease, Minneapolis, MN) to create an incomplete factorial matrix. A 2-factor interaction model of D-optimal design produced a matrix of 81 conditions (Table 2) that can be plated onto microtiter plates with 96 wells. This generic matrix was used as the primary screen for all of our test proteins.

Sample preparation

IL13

Recombinant human IL13 was expressed in *E. coli* strain BL21(DE3) as described previously [15]. Cells were resuspended in 50 mM Tris–Cl, pH 8.0, 1 mM EDTA, and lysed in a microfluidizer (Microfluidics, Newton, MA). The IL13 inclusion bodies were collected by centrifugation and washed two times with 0.1% Triton X-100 in 50 mM Tris–Cl, pH 8.8, 100 mM NaCl. The inclusion body protein was dissolved in 6 M guanidine–HCl, 50 mM Tris–Cl, 1 mM EDTA, and 20 mM DTT. The solution was cleared by centrifugation and passed through a NALGENE 0.45 μM membrane filtration unit (Nalge Nunc International, Rochester, NY) before refolding. The active control sample was a generous gift from Dr. Jim Wilhelm and was produced based on the published procedure [15].

MMP13

Escherichia coli cells BL21(DE3) expressing the procatalytic form of human MMP13 [16] were resuspended in 100 mM Tricine, pH 8.0, 5 mM EDTA, and 5 mM DTT, and lysed by passing through a Microfluidizer. The crude inclusion bodies containing MMP13 were collected by centrifugation and washed twice with 50 mM Tricine (pH 7.5), 100 mM NaCl, and 0.5% Triton X-100. The inclusion bodies were solubilized in 8 M urea, 50 mM Tricine, pH 7.5, 5 mM DTT, and further purified on Q-Sepharose under denaturing condition with a NaCl gradient. The folded, active control MMP13 used in the study was a generous gift from Dr. Jim Wilhelm [22].

LXRβ

A construct containing the LBD of rat LXRβ (amino acids 198–446) with a N-terminal 6×His-tag was used in this study. This construct was very similar to that used for the human LXRβ structure [20,21]. *E. coli* cells were resuspended in 50 mM Tris–Cl, pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol (β-Me), and lysed by passing through a Microfluidizer. The insoluble inclusion bodies were isolated by centrifugation and were solubilized in 8 M urea, 25 mM Tris–Cl, pH 8.0, and 10 mM β-Me and further purified on a Ni–NTA affinity column eluted with an imidazole gradient in the solubilization buffer. LXRβ containing fractions were pooled and used for further refolding. The soluble

Table 1
Parameters thought to affect protein refolding

Low concentration denaturants (A)	Buffers at different pH (B)	Detergents (C)	Divalent cations (D)
Urea[a]	NaOAc, pH 5.0[a]	Octyl glucoside (OG)[a]	EDTA[a]
GdmHCl[a]	Na/PO_4, pH 6.0	Lauryl Maltoside	$Cu^{2+}/Fe^{2+}/Mn^{2+}$
	Tris–Cl, pH 7.5[a]	CHAPS	$Mg^{2+}/Ca^{2+}/Zn^{2+}$
	Triethanolamine, pH 8.0	Sarkosyl	
	CHES, pH 9.0[a]	Triton X-100	
	Tris base, pH 10.0		
Salts (E)	**Aggregation suppressor/co-solvent (F)**	**Redox reagents**	
NaCl[a]	L-arginine[a]	DTT	
$(NH_4)_2SO_4$[a]	Glycerol[a]	2-Mercaptoethanol	
KCl[a]	PEG	Reduced/oxidized glutathione	
$KClO_4$	Sucrose	Cysteine/cystine	
$K_3Fe(CN)_6$	DMSO		
Imidazole	L-Gly		
$NaNO_3$	L-Pro		
LiCl	L-Lys		
	EtOH		

[a] Parameters included in the general matrix of 81 conditions.

Table 2
Conditions for general matrix I

Run	Denaturant	pH/buffers	Detergents	Divalent cations	Salts	Agg. supp.
1	1 M GdmHCl	5	0	0	0	0
2	2 M urea	5	0	0	0	0.4 M Arg
3	1 M GdmHCl	7.5	0.1% OG	0	100 mM KCl	10% glycerol
4	2 M urea	5	0	0	100 mM $(NH_4)_2SO_4$	10% glycerol
5	2 M urea	9	0	0	250 mM NaCl	10% glycerol
6	2 M urea	5	0.1% OG	0	250 mM NaCl	0.4 M Arg
7	0.5 M GdmHCl	5	0.1% OG	0	100 mM KCl	0.4 M Arg
8	1 M GdmHCl	5	0	0	250 mM NaCl	0.4 M Arg
9	1 M urea	7.5	0.1% OG	2 mM EDTA	100 mM KCl	10% glycerol
10	0.5 M GdmHCl	7.5	0.1% OG	0	100 mM $(NH_4)_2SO_4$	0
11	1 M urea	9	0.1% OG	2 mM EDTA	250 mM NaCl	0.4 M Arg
12	0	5	0	2 mM EDTA	100 mM KCl	10% glycerol
13	0.5 M GdmHCl	7.5	0.1% OG	0	250 mM NaCl	10% glycerol
14	1 M urea	9	0	2 mM EDTA	250 mM NaCl	10% glycerol
15	0	5	0	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0
16	2 M urea	9	0	0	100 mM KCl	0
17	2 M urea	9	0.1% OG	2 mM EDTA	0	0
18	1 M GdmHCl	9	0	2 mM EDTA	250 mM NaCl	0
19	1 M GdmHCl	7.5	0	0	100 mM $(NH_4)_2SO_4$	0.4 M Arg
20	1 M urea	9	0.1% OG	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0
21	1 M GdmHCl	7.5	0	2 mM EDTA	250 mM NaCl	10% glycerol
22	0	5	0.1% OG	0	100 mM KCl	0
23	0.5 M GdmHCl	9	0	0	100 mM $(NH_4)_2SO_4$	0.4 M Arg
24	0.5 M GdmHCl	7.5	0	0	0	0.4 M Arg
25	1 M urea	7.5	0	0	100 mM $(NH_4)_2SO_4$	10% glycerol
26	1 M GdmHCl	5	0.1% OG	2 mM EDTA	100 mM KCl	0.4 M Arg
27	0	9	0	0	100 mM KCl	0.4 M Arg
28	0.5 M GdmHCl	9	0	0	0	10% glycerol
29	2 M urea	7.5	0	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0
30	0.5 M GdmHCl	7.5	0	2 mM EDTA	250 mM NaCl	0
31	1 M GdmHCl	5	0.1% OG	0	100 mM $(NH_4)_2SO_4$	10% glycerol
32	2 M urea	7.5	0	0	100 mM KCl	0.4 M Arg
33	1 M GdmHCl	7.5	0.1% OG	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0
34	0	9	0	0	100 mM $(NH_4)_2SO_4$	0
35	0.5 M GdmHCl	5	0.1% OG	0	0	0
36	0.5 M GdmHCl	5	0	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0.4 M Arg
37	1 M urea	5	0	0	100 mM KCl	0.4 M Arg
38	0	9	0.1% OG	0	100 mM KCl	10% glycerol
39	1 M urea	7.5	0.1% OG	0	100 mM KCl	0
40	0.5 M GdmHCl	5	0.1% OG	2 mM EDTA	250 mM NaCl	0
41	2 M urea	5	0	2 mM EDTA	250 mM NaCl	0.4 M Arg
42	1 M GdmHCl	9	0	0	100 mM KCl	10% glycerol
43	2 M urea	9	0.1% OG	0	100 mM $(NH_4)_2SO_4$	10% glycerol
44	1 M urea	5	0.1% OG	2 mM EDTA	0	0
45	2 M urea	7.5	0.1% OG	0	0	0
46	0	9	0.1% OG	2 mM EDTA	250 mM NaCl	0
47	0	7.5	0	2 mM EDTA	0	0
48	0.5 M GdmHCl	9	0.1% OG	0	250 mM NaCl	0
49	0.5 M GdmHCl	9	0.1% OG	2 mM EDTA	0	0.4 M Arg
50	0	9	0.1% OG	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0.4 M Arg
51	0	7.5	0.1% OG	2 mM EDTA	250 mM NaCl	0.4 M Arg
52	0.5 M GdmHCl	7.5	0.1% OG	2 mM EDTA	100 mM KCl	0
53	2 M urea	5	0.1% OG	2 mM EDTA	250 mM NaCl	10% glycerol
54	1 M GdmHCl	9	0	2 mM EDTA	100 mM $(NH_4)_2SO_4$	10% glycerol
55	1 M GdmHCl	9	0.1% OG	2 mM EDTA	0	10% glycerol
56	0	7.5	0	0	100 mM KCl	10% glycerol
57	1 M GdmHCl	9	0.1% OG	0	100 mM KCl	0
58	0	7.5	0.1% OG	2 mM EDTA	100 mM $(NH_4)_2SO_4$	10% glycerol
59	1 M GdmHCl	9	0.1% OG	0	100 mM $(NH_4)_2SO_4$	0.4 M Arg
60	1 M GdmHCl	7.5	0.1% OG	0	250 mM NaCl	0
61	2 M urea	9	0.1% OG	2 mM EDTA	100 mM KCl	0.4 M Arg
62	1 M GdmHCl	7.5	0	2 mM EDTA	100 mM KCl	0
63	1 M GdmHCl	5	0	2 mM EDTA	0	10% glycerol

Table 2 (*continued*)

Run	Denaturant	pH/buffers	Detergents	Divalent cations	Salts	Agg. supp.
64	2 M urea	7.5	0.1% OG	2 mM EDTA	0	10% glycerol
65	0	7.5	0.1% OG	2 mM EDTA	100 mM KCl	0.4 M Arg
66	1 M GdmHCl	7.5	0.1% OG	0	0	0.4 M Arg
67	0.5 M GdmHCl	9	0	2 mM EDTA	100 mM KCl	10% glycerol
68	1 M urea	7.5	0	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0.4 M Arg
69	1 M urea	5	0.1% OG	0	100 mM $(NH_4)_2SO_4$	0.4 M Arg
70	0.5 M GdmHCl	5	0.1% OG	2 mM EDTA	100 mM $(NH_4)_2SO_4$	10% glycerol
71	0	5	0	0	250 mM NaCl	0
72	0	9	0	0	0	10% glycerol
73	1 M GdmHCl	9	0	2 mM EDTA	0	0.4 M Arg
74	0.5 M GdmHCl	5	0	0	100 mM KCl	0
75	1 M urea	5	0.1% OG	0	250 mM NaCl	0
76	2 M urea	7.5	0.1% OG	2 mM EDTA	250 mM NaCl	0
77	0	5	0.1% OG	0	0	0.4 M Arg
78	1 M urea	9	0.1% OG	0	0	0.4 M Arg
79	1 M urea	7.5	0.1% OG	2 mM EDTA	0	10% glycerol
80	1 M urea	7.5	0	0	250 mM NaCl	0
81	2 M urea	5	0.1% OG	2 mM EDTA	100 mM KCl	0

LBD control was isolated from the soluble *E. coli* lysate via Ni–NTA affinity and anion exchange columns. Detailed descriptions of the procedure will be published elsewhere.

Refolding reaction and dialysis

A 96-well polystyrene assay plate (Costar, Corning, Corning, NY) was used to carry out the refolding reaction. The protein of interest, solubilized in high concentration of denaturant, was 10–20-fold diluted into the 81 refolding buffers to give a final volume of 200 μl each with the final protein concentration at 100 μg/ml. In the case of IL13, each well was also dispensed with a redox mixture of 1:2 mM reduced/oxidized glutathione to facilitate disulfide bond formation. After dilution, the protein was incubated at room temperature for 30 min before it was transferred to a 96-well plate dialyzer (The Nest Group, Southborough, MA). The plate was capped and dialyzed against native buffer listed in the results section for each particular protein. Dialysis time ranged between overnight and 48 h at 4 °C with several manual inversions of the plate.

Detection

After dialysis, the samples were transferred to another 96-well plate and placed into the well plate autosampler of an Agilent1100 HPLC (Agilent Technologies, Wilmington, DE). A 100 μl sample from each well was injected directly onto a 2.3 × 30 mm Poros ion-exchange column (Applied Biosystems, Framingham, MA) equilibrated with the same buffer used for dialysis. The column was washed and developed with a NaCl gradient up to 1000 mM, with a flow rate of 0.5 ml/min over 25 column volumes. Protein eluted from the column was monitored by a fluorescence detector using an excitation at 230 nm and emission at 340 nm, based on the intrinsic tryptophan fluorescence of the protein. The column was regenerated with a 6 M guanidine–HCl wash at the end of each run. The overall configuration of the refolding plate assay is shown in Fig. 1.

Characterization of the refolded LXRβ

Because there has been no report on refolding of LXRβ in the literature, biophysical characterizations of our refolded material were carried out to further evaluate the conformational integrity of LXRβ. A Superdex 200 HR10/30 gel filtration column (GE Healthcare) was used to monitor the oligomeric state of the refolded LXRβ. The column was run in a buffer containing 25 mM Tris–Cl, pH 8.0, 200 mM NaCl, 5 mM DTT, and 100 nM T0901317 inhibitor compound [19], with a flow rate of 0.5 ml/min. The CD spectra in the region between 300 and 200 nm were analyzed to evaluate the amount of secondary structure content. All LXRβ CD samples were dialyzed against buffer containing 10 mM Tris–Cl,

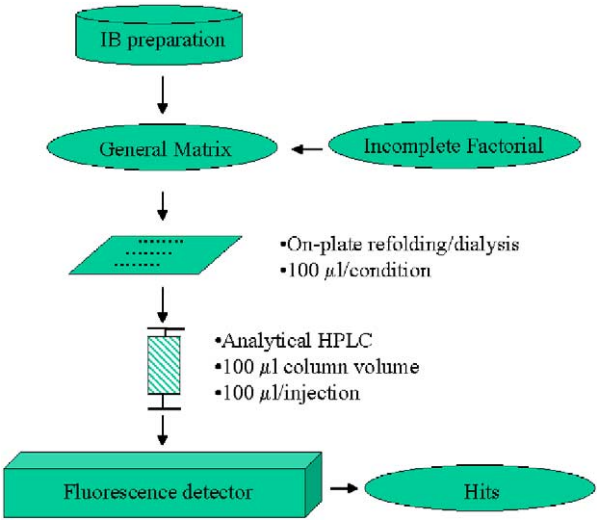

Fig. 1. Schematic drawing of the analytical refolding screen. IB, inclusion body.

pH 8.0, 1 mM DTT, 10 mM NaCl, and 1 μM T0901317 inhibitor compound. The CD spectra were recorded on a Jasco J-810 Spectropolarimeter. Because LXRβ is largely α-helical [20], the CD signal at 222 nm was monitored as a function of temperature to assess the thermal stability of the protein. An external Peltier type FDCD attachment (PFD 425 S) was used for temperature control, with a heating rate of 1 °C/min. The protein concentration was 0.5 μM and a 2.0 mm path length cell was used for all of the measurements.

Results

IL13 refolding

A published refolding protocol for IL13 was used as our positive control and refolding was performed in parallel with our 81 test conditions on the same 96-well plate (six replicates, condition #83 to #88). The plate was first dialyzed overnight against a buffer containing 50 mM CHES (pH 9.0), 100 mM NaCl to facilitate the formation of the disulfide bonds, and then overnight against 40 mM MES (pH 6.0), 20 mM NaCl, both at 4 °C. The predicted isoelectric point for IL-13 is 8.8, therefore a 2.3 × 30 mm Poros HS cation exchange column was chosen to separate the folded material from the misfolded species. The analytical Micro-S column was equilibrated with 40 mM MES (pH 6.0), 20 mM NaCl as the running buffer and elution was performed by a 20–1000 mM linear NaCl gradient. An aliquot of folded, active IL13 protein was used as a positive control on the Micro-S column. As shown in Fig. 2, the positive control condition (#84) gave a sharp elution peak at 5.4 min, which has the same retention time as the folded, active material, indicating that the separation/detection method is suitable for detecting the refolded IL13 material. All six-control conditions (#83 → #88) gave similar responses ranging from 19 to 23 Luminescent Units (LU) with #84 being closest to the average. All 81-matrix conditions were run on the same column with the same gradient and their elution profiles were evaluated. 17/81 conditions showed a detectable peak of refolded protein with condition #73 the highest (Fig. 2). The detailed composition for the top 10 conditions and their respective peak heights are listed in Table 3. Almost all 10 conditions showed higher

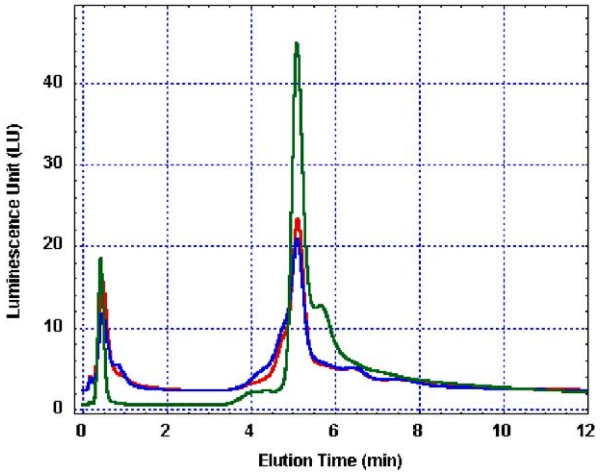

Fig. 2. Micro-S elution profile of IL-13. Green trace: a control sample of IL-13 prepared based on the published method by Moy et al. [15]. The protein was shown to be biologically active. Blue trace: condition #84, IL-13 refolded using the above method but in the same refolding plate as the 81 conditions. Red trace: condition #73 in the refolding assay. The major peaks of all three samples have the same elution time.

pH preference (9.0), even though pH 5.0, pH 7.5, and pH 9.0 were evenly distributed in the matrix. This is not surprising because disulfide bond formation is favored at alkaline pH. Guanidine–HCl is preferred over urea, even though there is an equal distribution of no denaturant, low concentration of urea, and low concentration of guanidine–HCl within the matrix of conditions tested. The presence of L-Arg is slightly preferred, while the presence of EDTA, detergent, and various salts did not seem to affect refolding yields. These results clearly suggest that this protein prefers to fold at basic pH, in the presence of low concentrations of guanidine–HCl, and possibly some L-Arg. Strikingly, this trend was shared by the published refolding condition for IL13 (3.0 M GdmHCl, 50 mM CHES, pH 9.0) [15], which was developed and optimized using conventional methods.

MMP13 refolding

The procatalytic form of MMP13 has been refolded from *E. coli* inclusion bodies and the structure of the catalytic domain has been solved using the refolded material

Table 3
Best conditions from the IL13 refolding assay

Run	Denaturant	pH	Detergents	Divalent	Salts	Agg. supp.	Response (LU)
73	1 M GdmHCl	9.0	0	2 mM EDTA	0	0.4 M Arg	22
59	1 M GdmHCl	9.0	0.1% OG	0	100 mM $(NH_4)_2SO_4$	0.4 M Arg	15
61	2 M urea	9.0	0.1% OG	2 mM EDTA	100 mM KCl	0.4 M Arg	14
54	1 M GdmHCl	9.0	0	2 mM EDTA	100 mM $(NH_4)_2SO_4$	10% glycerol	14
57	1 M GdmHCl	9.0	0.1% OG	0	100 mM KCl	0	13
78	1 M urea	9.0	0.1% OG	0	0	0.4 M Arg	13
23	0.5 M GdmHCl	9.0	0	0	100 mM $(NH_4)_2SO_4$	0.4 M Arg	12
24	0.5 M GdmHCl	7.5	0	0	0	0.4 M Arg	12
42	1 M GdmHCl	9.0	0	0	100 mM KCl	10% glycerol	12
49	0.5 M GdmHCl	9.0	0.1% OG	2 mM EDTA	0	0.4 M Arg	11
Control	3.2 M GdmHCl	9.0	0	0	0	0	21

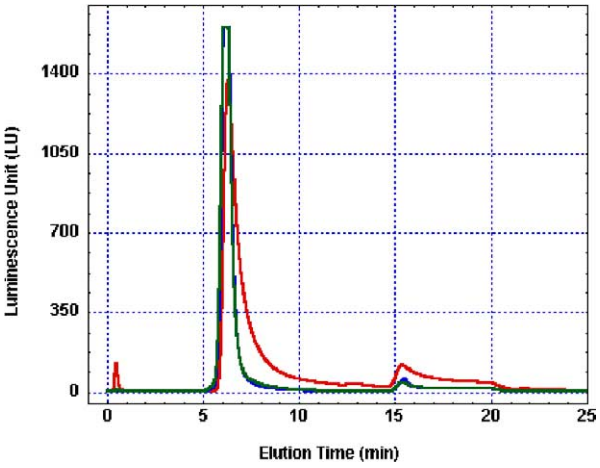

Fig. 3. Micro-S elution profile of MMP-13 procatalytic domain. Red trace: positive control. MMP-13 refolded based on the method by [16]. This method generated the procatalytic domain of MMP-13 that can be activated to produce the active MMP-13. Green trace: condition #84, control sample using the same method as the positive control but refolded on the same micro-titer plate as the 81 conditions. Blue trace: condition #19 in the refolding plate assay. The major peak from all three samples shares the same retention time on the Micro-S column.

[16,17]. We used the published refolding protocol as our positive control (#84). Its elution profile on the Micro-S column is shown in Fig. 3 (green trace). The predicted isoelectric point for the procatalytic MMP13 is 4.96. However, when tested for ion-exchange column binding, we found that the protein did not bind the HQ column (anion exchanger) at pH 7.5 but did bind to an HS column (cation exchanger) at pH 6.0, perhaps due to some unusual surface charge distribution on the protein. The elution time of condition #84 overlaps with the purified, refolded procatalytic MMP13 used for structure determination [17,22] (Fig. 3, red trace). Because MMP13 binds both Zn^{2+} and Ca^{2+}, the wells were supplemented with an additional 5 mM $CaCl_2$ and 10 μM $Zn(OAc)_2$. The resulting plate was dialyzed against 50 mM Tricine, pH 7.5, 400 mM NaCl, 5 mM $CaCl_2$, 100 μM $Zn(OAc)_2$, and 4 mM DTT once overnight and then against 40 mM MES, pH 6.0, 4 mM DTT, 5 mM $CaCl_2$, and 2 μM $Zn(OAc)_2$ overnight. Each condition was analyzed on a 2.3 × 30 mm Poros HS Micro-scale column with 40 mM MES, 5 mM $CaCl_2$, and 2 μM $Zn(OAc)_2$ as the running buffer. The column was developed with a 0–1000 mM linear NaCl gradient.

The procatalytic domain of MMP13 refolds very efficiently. Out of 81 conditions tested, only nine conditions resulting in undetectable refolding yields. All other conditions yielded some degree of folded material, with at least 13 conditions resulted in the highest refolding yields (>1600 LU), similar to the control (see Fig. 3, #19, for example). Table 4 shows the list of the 13 best refolding conditions for the MMP13 procatalytic domain. The majority (10/13) of the conditions are at pH 7.5, and the remainder are pH 9.0. All of the conditions that gave no detectable refolding are at pH 5.0, although the remainder of pH 5.0 conditions did show detectable levels of refolded protein. Based on the structure, the three histidine residues that coordinate the Zn^{2+} may be protonated at low pH, thus would be unable to coordinate the Zn^{2+}, which would hypothetically, reduce the refolding efficiency significantly [17,22]. Aggregation suppressors such as L-Arg, and co-solvents such as glycerol are not required for high refolding yields of MMP13. Almost all conditions have either low concentration of urea, or to a lesser extent, guanidine–HCl. The majority of the best conditions for refolding have some salt, although no preference is seen with three different salts tested. The presence or absence of detergents also did not seem to affect refolding yields. Overall, there was a strong correlation between the results we observed from the plate screen with the previously published refolding conditions for the MMP13 procatalytic domain, which is comprised of 50 mM Tricine, pH 7.5, 400 mM NaCl, 5 mM $CaCl_2$, 0.1 mM $Zn(OAc)_2$, and 4 mM DTT.

LXRβ refolding

When LXRβ is expressed in *E. coli*, a small fraction of the total protein is expressed in the soluble fraction and, although the yields are very low, folded and active protein

Table 4
Best conditions from the MMP13 Pro-Cat refolding assay

Run	Denaturant	pH	Detergents	Divalent	Salts	Agg. supp.	Response (LU)
16	2 M urea	9	0	0	100 mM KCl	0	>1600
17	2 M urea	9	0.1% OG	2 mM EDTA	0	0	>1600
19	1 M GdmHCl	7.5	0	0	100 mM $(NH_4)_2SO_4$	0.4 M Arg	>1600
29	2 M urea	7.5	0	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0	>1600
32	2 M urea	7.5	0	0	100 mM KCl	0.4 M Arg	>1600
39	1 M urea	7.5	0.1% OG	0	100 mM KCl	0	>1600
45	2 M urea	7.5	0.1% OG	0	0	0	>1600
46	0	9	0.1% OG	2 mM EDTA	250 mM NaCl	0	>1600
60	1 M GdmHCl	7.5	0.1% OG	0	250 mM NaCl	0	>1600
66	1 M GdmHCl	7.5	0.1% OG	0	0	0.4 M Arg	>1600
68	1 M urea	7.5	0	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0.4 M Arg	>1600
76	2 M urea	7.5	0.1% OG	2 mM EDTA	250 mM NaCl	0	>1600
80	1 M urea	7.5	0	0	250 mM NaCl	0	>1600
Control	0	7.5	0	0	400 mM NaCl	0	>1600

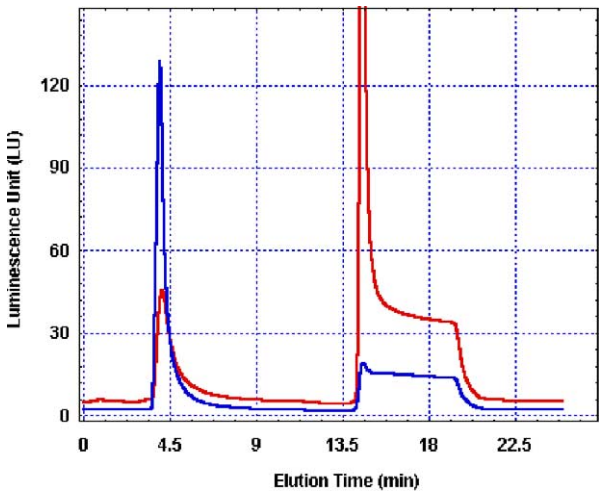

Fig. 4. Micro-Q elution profile of LXRβ LBD. Blue trace: 10 μg of soluble LXRβ LBD expressed and purified from *E. coli* used as positive control. Red trace: condition #27 in the refolding plate assay. Both samples were run in the presence of a known small molecule inhibitor T0901317 (1 μM).

can be purified from *E. coli* cleared lysates [18,19]. An efficient refolding protocol was desired because potentially, much more LXRβ could be generated from the inclusion body fraction, which would provide a significant advantage for drug discovery efforts, including structural biology. LXRβ has a predicted isoelectric point of 6.44, therefore an anion exchange column (Poros HQ) was the first choice for separation. LXRβ purified from *E. coli* cleared lysates (soluble fraction) was used as our positive control and its elution profile on the micro-scale Poros HQ (Micro-Q) column is shown in Fig. 4 (blue trace). The purified, solubilized inclusion bodies were diluted 20-fold into the 81 refolding solutions in a 96-well micro-titer plate and the plate was dialyzed overnight at 4 °C against buffer containing 25 mM Tris–Cl, pH 8.0, 5 mM DTT. Two refolding screens were done in parallel, one in the absence of ligand and the other in the presence of a known small molecule antagonist, T0901317 [19], at 10 μM. The inclusion of the second screen was based on the finding that, in the presence of a high affinity ligand during expression and purification, the yield of soluble and folded protein was dramatically increased for both Androgen Receptor (AR) and Progesterone Receptor (PR) LBDs [23,24]. Each refolding condition was analyzed on a Micro-Q column equilibrated with 20 mM Tris–Cl, pH 8.0, and the column was developed with a 0-1000 mM linear NaCl gradient. A number of refolding conditions showed a peak eluting at a similar NaCl concentration in the gradient as the soluble control. Overall, the conditions where folded LXRβ was detected were highly consistent between the two screens (+/−ligand) although the refolding yields were much higher in the screen that included the ligand, T0901317 (data not shown). The elution profile of the best condition, (#27, with ligand) as seen in Fig. 4 (red trace), was very similar to the LXRβ positive control. The detailed compositions of conditions resulting in the best refolding yields of LXRβ are listed in Table 5. None of the best refolding conditions contained guanidine–HCl, as the highest yields were obtained in either no denaturant or low concentrations of urea. Refolding yields at high pH (9.0) were slightly better than at pH 7.5, whereas yields at pH 5.0 were significantly lower. There was no clear trend in refolding yields for the presence or absence of detergents. The inclusion of a metal chelator, EDTA, had no obvious impact on refolding yields, nor did any specific type of salt, but the addition of salt strongly correlated with LXRβ refolding yields. The preference of L-Arg over glycerol remains to be determined, since only the top two conditions have L-Arg and the rest mostly contain glycerol. We were unable to compare our refolding conditions with any known condition since the refolding of the LXRβ LBD has not been documented in the literature. The refolding experiments were repeated two more times in order to compare the reproducibility of our data. The best hit condition, #27, remained within the top five hits, although the ranking order was somewhat different (data not shown). The general trend of the refolding condition for LXRβ remained the same.

LXRβ scale-up refolding and characterization

The refolded LXRβ LBD behaved similarly as the *E. coli* expressed soluble control on the anion exchange column. However, because our refolding protocol for LXRβLBD is novel, we want to provide additional evidence to further demonstrate the structural integrity of the refolded material. A bioactivity assay was not suitable since the conditions that produced much higher refolding yields also included the antagonist, T0901317. Instead, we carried out biophysical analysis to characterize the refolded LXRβ

Table 5
Best conditions from the LXRβ LBD refolding assay

Run	Denaturant	pH	Detergents	Divalent	Salts	Agg. supp.	Response (LU)
27	0	9	0	0	100 mM KCl	0.4 M Arg	46
50	0	9	0.1% OG	2 mM EDTA	100 mM $(NH_4)_2SO_4$	0.4 M Arg	38
39	1 M urea	7.5	0.1% OG	0	100 mM KCl	0	28
14	1 M urea	9	0	2 mM EDTA	250 mM NaCl	10% glycerol	27
25	1 M urea	7.5	0	0	100 mM $(NH_4)_2SO_4$	10% glycerol	26
46	0	9	0.1% OG	2 mM EDTA	250 mM NaCl	0	26
5	2 M urea	9	0	0	250 mM NaCl	10% glycerol	25
58	0	7.5	0.1% OG	2 mM EDTA	100 mM $(NH_4)_2SO_4$	10% glycerol	25
43	2 M urea	9	0.1% OG	0	100 mM $(NH_4)_2SO_4$	10% glycerol	22

LBD. To obtain enough protein for our study, a 10 mg scale-up refolding reaction was carried out using condition #27 as the test case (Table 5). A typical chromatograph of the LBD on a Poros HQ anion exchange column is shown in Fig. 5. Refolded LXRβ LBD elutes as a sharp peak early in the NaCl gradient. This behavior is similar to that of the active material expressed in the soluble fraction. Some misfolded material eluted later during the gradient as a shoulder behind the main peak. The overall refolding yield after the HQ column step was ~15%, which is quite reasonable without any attempt at optimization. An aliquot of the HQ eluate was loaded onto a Superdex 200 gel filtration column to evaluate its oligomeric state. As shown in Fig. 6A, the refolded material has the identical elution profile as the soluble control. No aggregation was observed, suggesting that the refolded material exists as a single species in solution, identical to that of the soluble LXRβ LBD.

The refolded LXRβ LBD was further characterized by collecting the CD spectra of the protein in the ultraviolet region near 200 nm, where the peptide bond absorbs and secondary structure elements have well-defined spectra [25]. Furthermore, the stability of the HQ purified material was determined by thermal denaturation. As shown in Fig. 6B, the refolded LXRβ has the same CD spectra as the control. Both samples showed largely α helical content with a double minima at 208 and 222 nm, which is consistent with its X-ray structure [20,21]. Fig. 6C shows the melting curve of the refolded LXRβ, together with the soluble control, as monitored by CD at 222 nm. Both samples exhibited virtually identical melting temperatures, as the refolded LXRβ was 49.8 °C, while the T_m for the soluble control was 49.5 °C. The high cooperativity during denaturation is often a hallmark of protein unfolding from a native state [26]. Taken together, our data indicated that our refolded LXRβ LBD shares identical biophysical properties as the active control.

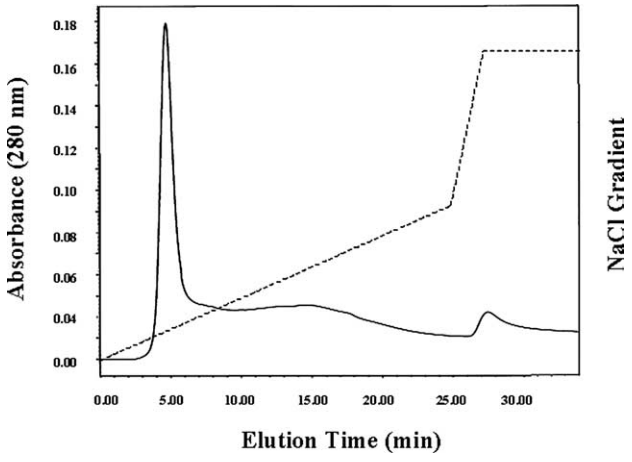

Fig. 5. Scale-up refolding of LXRβ LBD using condition #27 in the presence of the small molecule ligand T0901317. The overall recovery of refolding is ~1.5 mg with 10 mg of unfolded LXRβ LBD in 8 M urea as the starting material. The protein concentration during refolding is 50 μg/ml. A 1.7 ml Poros HQ column was used for this experiment.

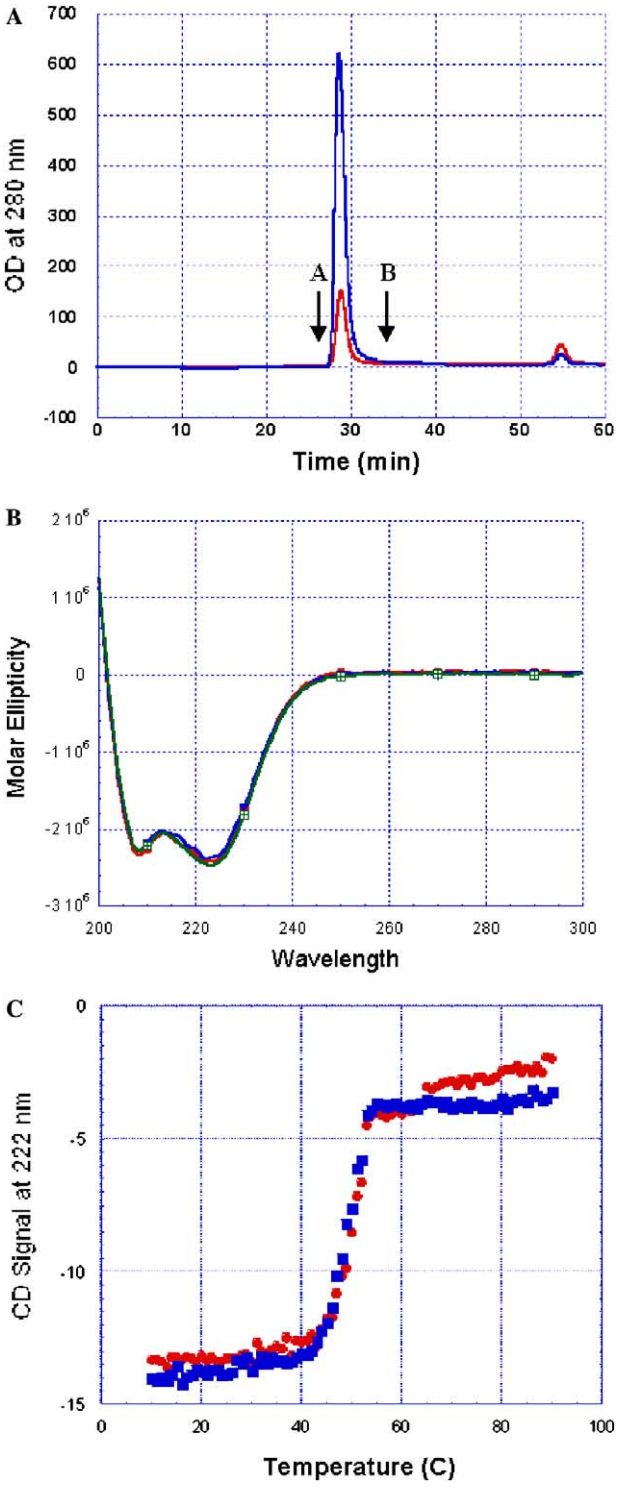

Fig. 6. (A) Gel filtration chromatography profile of LXRβ LBD. Blue: soluble control. Red: an aliquot of refolded LXRβ from the HQ elution of the scale-up refolding experiment. The column (Superdex 200) was developed with buffer containing 25 mM Tris–Cl, pH 8.0, 200 mM NaCl, 5 mM DTT, and 100 nM T0901317. The flow rate was 0.5 ml/min. The arrows indicate the retention time of the molecular weight standards. A: chicken ovalbumin (44 kDa) and B: horse myoglobin (17 kDa). (B) Far-UV CD spectra of LXRβ LBD. Green: soluble control. Blue: refolded LXRβ from the HQ elution. Both spectra were normalized to molar ellipticity for comparison. (C) Thermal denaturation of LXRβ LBD. The CD signal at 222 nm was monitored as a function of temperature. Blue square: soluble control. Red circle: refolded LXRβ from HQ column elution.

Discussion

Recombinant proteins are essential drug discovery tools for assay development, high-throughput screening, and structural biology efforts. In the post-genomic era, translation of thousands of ORFs into milligram quantities of active proteins is a major challenge. The easiest way to obtain the target protein in a significant quantity in a short time frame is by over-expressing it in a heterologous system such as *E. coli*. Frequently, this approach has yielded expression of fully functional proteins. However, in many cases, expressing the protein of interest in *E. coli* results in the formation of insoluble inclusion bodies. Technologies in protein refolding that improve both the throughput and the success rate in generating active protein material are needed. The pioneering work done by Anfinsen and coworkers [27,28] and later by other groups showed that, in principle, the amino acid sequence of a protein contains all the necessary information to fold into a functional three-dimensional structure. However, the exact rules of how a protein sequence determines the final structure still remain unclear. This "protein-folding problem" has been the topic of intense research for the past several decades. Meanwhile, developing a protocol to refold a recombinant protein for further study has remained largely empirical. Very often, the folding conditions for one protein cannot be directly applied to others and furthermore, identifying folding conditions with reasonable yields for each protein involves an exhaustive screen of many conditions even for a small protein such as insulin-like growth factor I [29]. Traditionally, when screening for refolding conditions, a functional assay is used to assess the yields. Ideally, the assay should be quantitative, simple to execute, and sensitive enough to detect less than 1% yields at about 100 μg/ml. Certainly, there are many proteins including orphan nuclear receptors, novel protein kinases, and growth factors where either substrates or ligands have not been identified and of course, novel proteins of unknown function where defining and developing a possible assay for screening a large number of refolding conditions may be quite difficult. The refolding assay we have developed here is a significant step in overcoming these obstacles. The incomplete factorial approach allows us to sample a large number of conditions simultaneously, the 96-well plate format for refolding and dialysis is amenable to full automation, fluorescence detection requires a minimal amount of protein and is highly sensitive to detect very low refolding yields (<1%), and the automatic sample injection system allows complete analysis within two days. Furthermore, the chromatographic separation of folded from misfolded protein overcomes the limitation on the availability of a functional assay. The complete assay analysis requires only 4–5 days to run, including sample preparation, dialysis, and chromatographic analysis, which is much more rapid than traditional approaches that can take up to a month to test the same number of conditions. By using this method, one can quickly determine whether a protein target can be refolded from *E. coli* inclusion bodies before devoting time and effort pursuing other expression systems.

We have tested three proteins in our refolding assay. IL13, MMP13 procatalytic domain, and LXRβ LBD have quite different molecular properties including molecular weight, presence or absence of disulfide bonds, calculated p*I*, and overall fold. All of them showed some degree of refolding. Two of them, IL13 and MMP13, have been refolded previously and our refolding system identified conditions that correlate very well with known conditions. The third protein, LXRβ LBD, gave refolding yields of ~15% upon scaling the process. Because refolding of the LBD has not been described in the literature to date and a bioactivity assay to characterize the refolded material was not possible in the presence of an inhibitor, we have characterized the refolded LBD by additional biophysical experiments. Our results showed that the refolded LXRβ LBD and the soluble control LBD share virtually identical biophysical properties including the same profile on a size exclusion column, identical CD spectra, and an overlapping, highly cooperative thermal denaturation curve, indicating that the two share very similar conformations and are well-folded proteins. The requirement for ligand during refolding for LXRβ is not without precedent. The expression and purification of the LBD of the PR in *E. coli* requires the addition of progesterone both during induction and purification [23]. Successfully, crystallizing and solving the X-ray structure of the androgen receptor LBD in complex with the ligand, metribolone, required the addition of ligand during *E. coli* cell induction and purification [24]. There are examples, such as estrogen receptors α and β, where the LBD can be expressed as a soluble, active protein in *E. coli* [30,31] and purified to homogeneity but can only be crystallized in the presence of a small molecule ligand. The LXRβ LBD most likely belongs to this category [20]. The requirement of the ligand for structure determination seems to be a general trend for the steroid and nuclear receptors and this might suggest a conformational flexibility that is intrinsic to this family of the proteins without ligand. In fact, a recent study on the refolding of the glucocorticoid receptor (GR) indicated that GR can be refolded into a compact, native-like structure in the absence of a ligand but this refolded material binds the ligand dexamethasone much weaker (13,000-fold less) than the in vivo assembled receptor [32]. A thorough biophysical analysis was carried out to characterize this refolded GR although the in vivo folded counterpart was not used for comparison. The authors concluded that the refolded GR in the absence of its ligand, although native-like, is different from the in vivo expressed, high affinity ligand-binding state, which most likely is stabilized by its association with several molecular chaperones and cofactor proteins [33]. It is worthwhile to note that in contrast to the LXR thermal denaturation profile shown here, the reported guanidine–HCl denaturation transition for the refolded GR was much less cooperative. The presence of a nondenaturing detergent was necessary to maintain this refolded material in a soluble, nonaggregated state. Our in vitro refolding study of LXRβ LBD demonstrated that the presence of a proper

ligand greatly increases the folding efficiency and, potentially, direct the receptor to fold into a more stable conformational state that would no longer require the interaction with the chaperone system or detergent.

Our refolding assay has some limitations. The fluorescence detector relies on the presence of tryptophan residues in the protein sequence. Although this is not an issue with most proteins, there are some that have no tryptophan. In this case, it might be necessary to engineer one or more Trp residues into the protein sequence as an N or C-terminal extension (such as the Strep-tag affinity purification system), or alternatively, one can introduce a post-column modification step to uniformly label the protein with a non-specific dye for detection, which is being used routinely in the field of amino acid analysis, for example. Another concern we have is the pH range we included in our screen. Although it has been reported in the literature that some proteins can be refolded at a pH as low as 5.5 [34], we have not seen many refolding hits from the low pH conditions, regardless whether or not disulfide bonds are present in the protein. A similar observation is shared by the study done in Gouaux' group [9]. We are in the process of modifying our screen so that a higher pH range (such as pH 7–10) is included in order to be more representative of the common refolding conditions for most proteins. Additionally, for a novel protein, it might not be straightforward to identify a proper column to be used to separate folded material from the misfolded species. In our experience, we are most successful by trying the ion-exchange columns first, based on the predicted p*I* value of the protein. In some cases, a size exclusion column has been used. A folded control is convenient to have for calibration of the elution profile but is not absolutely necessary. Lastly, although we have not encountered such a problem, it has been reported in the literature that refolding of some proteins including human insulin-like growth factors [35,36] produce a misfolded minor species that give reasonably well-behaved chromatographic profiles. In these rare cases, one should use other methods of analysis such as an activity assay to confirm the integrity of the refolded proteins.

In summary, we have demonstrated the feasibility and applicability of a refolding assay that is fully automatable using a non-functional assay for detection. We have successfully applied this approach to several model proteins shown in this study, as well as a number of protein targets in our laboratory, including β-secretase, interleukin 21, and tropomyosin receptor kinase domain 5. This methodology can be used potentially in high-throughput structural analysis for drug discovery efforts.

Acknowledgments

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