

A high-throughput protein refolding screen in 96-well format combined with design of experiments to optimize the refolding conditions

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ABSTRACT

Production of correctly folded and biologically active proteins in *Escherichia coli* can be a challenging process. Frequently, proteins are recovered as insoluble inclusion bodies and need to be denatured and refolded into the correct structure. To address this, a refolding screening process based on a 96-well assay format supported by design of experiments (DOE) was developed for identification of optimal refolding conditions. After a first generic screen of 96 different refolding conditions the parameters that produced the best yield were further explored in a focused DOE-based screen. The refolding efficiency and the quality of the refolded protein were analyzed by RP-HPLC and SDS-PAGE. The results were analyzed by the DOE software to identify the optimal concentrations of the critical additives. The optimal refolding conditions suggested by DOE were verified in medium-scale refolding tests, which confirmed the reliability of the predictions. Finally, the refolded protein was purified and its biological activity was tested *in vitro*. The screen was applied for the refolding of Interleukin 17F (IL-17F), stromal-cell-derived factor-1 (SDF-1 α /CXCL12), B cell-attracting chemokine 1 (BCA-1/CXCL13), granulocyte macrophage colony stimulating factor (GM-CSF) and the complement factor C5a. This procedure identified refolding conditions for all the tested proteins. For the proteins where refolding conditions were already available, the optimized conditions identified in the screening process increased the yields between 50% and 100%. Thus, the method described herein is a useful tool to determine the feasibility of refolding and to identify high-yield scalable refolding conditions optimized for each individual protein.

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Introduction

Functional and structural studies of proteins often require large amounts of correctly folded, pure, and active protein. Bacterial expression systems, mostly in *Escherichia coli*, are cheap, simple and frequently used to produce large quantities of recombinant proteins. However, production of foreign proteins in *E. coli* frequently leads to the formation of inclusion bodies (IB).¹ Inclusion

bodies are insoluble protein aggregates composed of precipitated unfolded or incorrectly folded protein molecules devoid of biological activity. The major challenge is to efficiently convert this inactive and insoluble protein into soluble, correctly folded and active product [1,2]. To achieve this, the protein needs to be submitted to an elaborated solubilization, refolding and purification procedure [3–5]. After a complete reduction and linearization of the protein from the inclusion bodies, the *in vitro* refolding involves several crucial steps. The covalent disulfide bonds must be generated correctly and the molecule must adopt the native secondary, tertiary, and quaternary structure. Defining conditions that promote refolding of a chemically denatured protein into its native conformation is often empirical, time consuming and frequently yields low amounts of correctly refolded protein.

In recent years, several high-throughput protein refolding methods have been developed [6,7]. These methods are based on dilution, dialysis or solid phase immobilization for the renaturation process [8]. Dialysis and dilution based methods along with the use of various additives have been reported to improve recovery of

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¹ Abbreviations used: DOE, design of experiments; IL-17F, interleukin 17F; SDF-1 α /CXCL12, stromal-cell-derived factor-1; BCA-1/CXCL13, B cell-attracting chemokine 1; GM-CSF, granulocyte macrophage colony stimulating factor; SPA, scintillation proximity assay; SEC, size exclusion chromatography; IB, inclusion bodies; PEG, polyethylene glycol; DTT, dithiothreitol; IPTG, isopropyl- β -D-thiogalactopyranoside; TFA, trifluoroacetic acid; FCS, fetal calf serum; PI3K gamma, phosphatidylinositol-3 kinase gamma.

refolded proteins [6]. Other methods described to increase refolding yields include pulse renaturation processes [9], size exclusion chromatography and adsorption chromatography [10]. However, identifying the optimal refolding conditions can be difficult and time consuming.

Several fractional factorial refolding screens (Novagen iFOLD™, Hampton Research Foldit™, and AthenaES QuickFold™) are available commercially [11]. Despite this, several difficulties still remain. The screens only identify additives affecting the refolding, but optimal concentrations and cooperative interactions between additives are not determined. Furthermore, the method for determination of refolding yields is frequently not well defined and/or unreliable.

Different publications have shown that physical parameters such as pH, ionic strength, and temperature may have a tremendous effect on the efficacy of the *in vitro* folding process [12]. Aggregation is presumably the major process competing with correct refolding. Therefore a logical strategy to improve refolding yield is to prevent aggregation by adding low molecular weight compounds that interfere with unwanted protein–protein interactions.

A wide range of chemical additives have been described to prevent or reduce misfolding of proteins during the refolding process. L-Arginine is one of the most widely used additives for refolding of proteins. Its effects as enhancer of *in vitro* protein refolding were discovered a decade ago [1]. L-Arginine, which contains a guanido group, does not destabilize the native folded structure to the same extent as guanidine chloride, and at a concentration between 0.2 and 1 M, frequently increases the renaturation yield significantly [13]. An additional mechanism of L-arginine as suppressor of aggregation was recently proposed [14].

Polyethylene glycol (PEG) is another additive frequently used to improve correct structure formation by inhibiting aggregation [15]. Several studies have suggested that the success of PEG as a refolding additive depends on maintaining a specific molar ratio of PEG to protein [16]. Salt concentration plays an important role for the solubility of the protein during refolding. Mostly, NaCl or KCl is used at concentrations between 0 and 500 mM. For several proteins, nonionic as well as ionic and zwitterionic detergents have been found to have a favorable effect on the renaturation process [17]. Very efficient protein folding has been achieved by using a mixture of detergents (e.g. Triton X-100 or lauryl-maltoside) and phospholipids [18]. Non-detergent sulfobetaines (NDSBs) have shown to have a positive synergistic interaction with reductants and to decrease protein aggregation [19].

Proteins that possess disulfide bonds present additional challenges, since the folding of the protein is dependent upon correct disulfide bond formation. Disulfides formed between the wrong residues lead to aggregated or misfolded proteins. A low concentration of EDTA is frequently recommended to prevent metal-catalyzed air oxidation of cysteines, which could result in wrong disulfide bridge formation [5]. Addition of a mixture of reduced (RS^-) and oxidized ($RSSR$) forms of low molecular weight thiol reagents such as glutathione (GSH/GSSG), cysteine/cystine, cystamine/cysteamine and dithiothreitol (DTT/ODTT) usually provides the appropriate redox potential to allow formation and reshuffling of correct disulfide bridges [20]. The mechanism of disulfide bond formation has been treated by Gilbert [21]. The optimal ratio of the redox mixture in the refolding buffer is protein-specific. The ratio of GSH to GSSG should correspond to a redox potential (E_0) that is compatible with protein disulfide formation [22]. For refolding experiments, various ratios of reduced to oxidized reagents at 0.2–5 mM are frequently used.

Protein concentration has a major impact on the refolding yield and is one of the key factors for successful renaturation. Because of the increased risk of aggregation at high protein concentrations, renaturation often has to be performed at a high dilution [23].

However, refolding at low concentrations is generally not economical for large scale protein production. Thus, many proteins can be folded at concentration near 1 mg/ml under optimized conditions [24,25].

Recently, the design of experiments (DOE) approach was used to optimize the refolding of a human cytokine fusion protein from *E. coli* inclusions bodies [26]. The DOE approach allows for the evaluation of the effect of additives over a wide concentration range, as well as their interactions, in a very efficient manner. This tool was successfully used to improve the refolding yield by 57% for the protein progenipointin-1 [26]. By using this approach, interactions between the two additives cysteine and urea were identified, which could not be detected with an empirical refolding process.

There is no rational method for predicting conditions that will promote the refolding of any given protein. In this study, we have developed a generic 96-well refolding screen combined with a protein specific DOE-based focused secondary screen. The refolding yield was determined by a generic RP-HPLC analytical method for identification of the optimal refolding conditions. To set-up and validate the refolding screen, five different proteins were used, interleukin 17-F (IL-17F) which is a 30 kDa disulfide-linked homodimer, stromal-cell-derived factor-1 (SDF-1 α /CXCL12), an 8-kDa CXC chemokine, B cell attractant chemokine 1 (BCA-1/CXCL13), anaphylatoxin C5a, a small protein of 74 residues [27–30] and granulocyte macrophage colony stimulating factor (GM-CSF), a 15 kDa cytokine [31,32].

Materials and methods

Materials and reagents

Tris (hydroxymethyl) aminomethane, D-sucrose, L-arginine, tri-sodium citrate, citric-acid, sodium chloride, polyethylene glycol 3300, and dithiothreitol (DTT) were obtained from Fluka. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Acros Organics. Zwittergent 3-14, ethanolamine and NDSB-256 were from Calbiochem. Guanidium hydrochloride was purchased from ABCR GmbH & Co. Source 30 RPC chromatography media, columns and the AKTA Explorer 100 equipped with an air detector were from GE-Healthcare Lifesciences. SE-HiCap Fractogel (M) was supplied by Merck KGaA. Multi Screen Deep Well Solvintert low binding Hydrophilic PTFE plates were from Millipore. The Cornerstone Design of experiment software (version 4.2.2) was purchased from P&P Informations technologie GmbH.

Protein expression and preparation of inclusion body extracts

All proteins described in this paper were expressed in *E. coli* and extracts prepared following the protocol below. The expression vectors were derived from the pET30 backbone vector (Novagen) and carried the gene of interest downstream of the T7/lac promoter. The plasmids were transformed into competent BL21 [DE3] *E. coli* cells. 5 L of culture medium containing 0.1 mM kanamycin were inoculated and, after induction with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at $OD_{600} = 10$, grown for 3.5 h at 37 °C. The cells were harvested and centrifuged at 10,000 $\times g$ for 60 min at 4 °C (RC5C centrifuge, Sorvall instruments). Wet cell paste (100 g) was suspended in 500 ml 100 mM Tris–HCl buffer pH 8.0 containing 20 mg/L of DNase. Cells were broken by three passages through a French Press at 1600 bars. The suspension was centrifuged at 10,000 $\times g$ for 60 min at 4 °C (RC5C centrifuge). The inclusion body pellet containing the recombinant protein was washed with 500 ml water two times, solubilized in 500 ml 0.1 M Tris/HCl, pH 8.0, containing 6 M guanidine/HCl and 10 mM

DTT, stirred for 30 min at 60 °C and centrifuged at 100,000×g for 60 min (Beckman Optima L-100). The soluble fraction containing the denatured protein of interest was filtered on a 0.2 µm filter and captured on a 192 ml Source 30 RPC column (Fine line 3.6/20) equilibrated in 0.1% trifluoroacetic acid (TFA) in water at a flow rate of 10 ml/min. Proteins were eluted with a 0–100% gradient of acetonitrile in 0.1% TFA in water. Fractions containing the protein of interest were pooled and lyophilized. The protein was solubilized at 4 mg/ml in 0.1 M Tris/HCl, pH 7.5, containing 6 M guanidine/HCl and 2 mM DTT.

Determination of protein concentration

Protein concentrations were determined from the absorbance at 280 nm using the following extinction coefficients (ϵ) 14,660 M⁻¹ cm⁻¹ for Met-IL-17F, 8730 M⁻¹ cm⁻¹ for Met-SDF-1 α , 18,830 M⁻¹ cm⁻¹ for Met-BCA-1, 11,290 M⁻¹ cm⁻¹ for Met-GM-CSF and 3400 M⁻¹ cm⁻¹ for Met-6HIS-C5a.

Preparation of the master buffer plate for 96-well generic screening plate

The master buffer plate was prepared in-house with an AKTA explorer 100 chromatographic system. The stock solutions used to prepare the master buffer plate are as follows.

0.91 M Citrate/citric-acid, pH 6.5, 0.91 M citrate/citric-acid pH 6.5 + 0.91 M L-arginine, 0.91 M Tris/HCl pH 7.5, 0.91 M Tris/HCl pH 7.5 + 0.91 M L-arginine, 0.91 M Tris/HCl pH 8.5, 0.91 M Tris/HCl pH 8.5 + 0.91 M L-arginine, 0.91 M ethanolamine/HCl pH 9.5, 0.91 M ethanolamine/HCl pH 9.5 + 0.91 M L-arginine, 0.5 M NaCl, 1 M sucrose, 2.3 M NDSB 256, 0.25% (w/v) PEG 3300, 0.1% Zwittergent 3-14, 1.5 mM/15 mM reduced/oxidized glutathione, and 15 mM/1.5 mM reduced/oxidized glutathione.

Each stock solution was connected to a buffer line on the chromatographic system purged with the corresponding solution. To prepare the generic master plate, a program was set-up to distribute stock solutions directly into an empty 96 deep well plate loaded on the fraction collector. The stock solutions were mixed into the wells of the plate according to the preestablished template and at the end of the preparation the plate contains a final volume of 2 ml in each well (Fig. 1). All master plates in this study were freshly prepared and used immediately to avoid possible problems of stability of any additives.

The final concentrations in the master buffer plate are as follows.

0.5 M Citrate/citric-acid pH 6.5, 0.5 M Tris/HCl pH 7.5, 0.5 M Tris/HCl pH 8.5 and 0.5 M ethanolamine/HCl pH 9.5, 0.5 M L-arginine, 100 mM NaCl, 0.2 M D-sucrose, 0.46 M NDSB 256, 0.05% PEG 3300 and 0.02% Zwittergent 3-14. Two ratios of reduced and oxidized glutathione were used: 3 mM / 0.3 mM and 0.3 mM / 3 mM (Fig. 1).

Primary generic refolding screen in 96-well plate

The denatured target protein at 4 mg/ml was refolded by dilution (1:20). One-hundred microliters of the denatured protein solution was added into each well of a 96-deep well plate at the beginning of the refolding process. The refolding buffers from the master buffer plate were transferred in 4 pulses (100, 200, 400 and 1200 µl) into the plate containing the denatured protein every 15 min during 1 h. Each of the first 3 pulses correspond to a two-fold dilution which permitted to slow down the refolding process compared to the drop wise dilution process frequently used. At the end of the refolding each well contains a final volume of 2 ml with the protein at a concentration of 0.2 mg/ml. This concentration could be considered high but was chosen to reduce the volume during refolding later at pilot scale and to facilitate analysis of the

	1	2	3	4	5	6	7	8	9	10	11	12		
A	NDSB256	Buffer only	PEG 3300	NaCl	ZWITT 3-14	Sucrose	ARG without GSH/GSSG	ARG	ARG + PEG 3300	ARG + NaCl	ARG + ZWITT 3-14	ARG + Sucrose	GSH/GSSG (3/0.3 mM)	Citrate 0.5 M pH 6.5
B	Buffer without GSH/GSSG	Buffer only	PEG 3300	NaCl	ZWITT 3-14	Sucrose	NDSB256	ARG	ARG + PEG 3300	ARG + NaCl	ARG + ZWITT 3-14	ARG + Sucrose	GSH/GSSG (0.3/3 mM)	
C	NDSB256	Buffer only	PEG 3300	NaCl	ZWITT 3-14	Sucrose	ARG without GSH/GSSG	ARG	ARG + PEG 3300	ARG + NaCl	ARG + ZWITT 3-14	ARG + Sucrose	GSH/GSSG (3/0.3 mM)	Tris 0.5 M pH 7.5
D	Buffer without GSH/GSSG	Buffer only	PEG 3300	NaCl	ZWITT 3-14	Sucrose	NDSB256	ARG	ARG + PEG 3300	ARG + NaCl	ARG + ZWITT 3-14	ARG + Sucrose	GSH/GSSG (0.3/3 mM)	
E	NDSB256	Buffer only	PEG 3300	NaCl	ZWITT 3-14	Sucrose	ARG without GSH/GSSG	ARG	ARG + PEG 3300	ARG + NaCl	ARG + ZWITT 3-14	ARG + Sucrose	GSH/GSSG (3/0.3 mM)	Tris 0.5 M pH 8.5
F	Buffer without GSH/GSSG	Buffer only	PEG 3300	NaCl	ZWITT 3-14	Sucrose	NDSB256	ARG	ARG + PEG 3300	ARG + NaCl	ARG + ZWITT 3-14	ARG + Sucrose	GSH/GSSG (0.3/3 mM)	
G	NDSB256	Buffer only	PEG 3300	NaCl	ZWITT 3-14	Sucrose	ARG without GSH/GSSG	ARG	ARG + PEG 3300	ARG + NaCl	ARG + ZWITT 3-14	ARG + Sucrose	GSH/GSSG (3/0.3 mM)	EtOHNH ₂ 0.5 M pH 9.5
H	Buffer without GSH/GSSG	Buffer only	PEG 3300	NaCl	ZWITT 3-14	Sucrose	NDSB256	ARG	ARG + PEG 3300	ARG + NaCl	ARG + ZWITT 3-14	ARG + Sucrose	GSH/GSSG (0.3/3 mM)	

Fig. 1. Primary 96-well generic screening plate. The plate is divided vertically into two parts, the left section corresponds to conditions without L-arginine (columns 1–6), whereas the wells in the right section contain 0.5 M L-arginine (columns 7–12). Horizontally, the plate is divided into four sections with different pH from 6.5 (row A–B) to pH 9.5 (row G–H). Each buffer has two rows the upper under reducing conditions with a ratio of 3/0.3 mM of GSH/GHG and the lower under oxidizing conditions with a ratio of 0.3/3 mM of GSH/GHG. Other additives are as indicated in the figure. All additives were tested at one single concentration as indicated in the text.

refolded proteins. After the dilution the refolding plate was mixed on a plate shaker over night at 4 °C. The 96 samples were filtered on a MultiScreen Deep Well Solvint low binding hydrophilic PTFE plate (Millipore, Ref. MDRLN0410) equipped with a 0.45 µm filter at the bottom. The protein concentration in the filtrates was estimated by measuring the optical density at 280 nm.

Analysis of refolding yield and quality

HPLC

RP-HPLC was used to verify the correct folding of the protein. A VYDAC C-4 column (3 µm, 50 mm × 4.6 mm) was equilibrated in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. 500 µl filtered protein samples, prepared as described above, were injected onto the column and proteins were eluted with a linear gradient from 35% to 72% acetonitrile in 0.1% TFA during 12 column volumes. The elution profile monitored at 214 nm was compared to standard runs with the correctly refolded protein and the unfolded and reduced protein in 6 M GuHCl/10 mM DTT. Elution fractions containing the refolded protein were pooled, lyophilized and solubilized in 100 µl 0.2 M Tris–HCl, pH 7.5. The amount of refolded protein was determined by measuring the optical density at 280 nm. The refolding yield was calculated based on the total protein amount used for the refolding.

Electrophoresis

SDS–PAGE was used to assess the purity of the refolded protein. In addition, for some proteins like IL-17F which is a homodimer linked together by 2 inter disulfide bridges, the refolding efficiency could be verified easily by comparing reduced and non-reduced conditions. Proteins were analyzed on a 10% polyacrylamide gel (NuPAGE 10% Bis-Tris Gel 1.0 mm, 10 wells from Invitrogen) and stained with Coomassie Simply blue SafeStain (Invitrogen). The gels were scanned on a BioRad GS-800 densitometer and the band intensities were determined using the Quantity one software.

Optimization of the refolding conditions by design of experiments (DOE)

The results from the primary screen indicated the best pH for the refolding as well as the additives which have a favorable effect on the refolding. Based on the results from the primary screen the DOE software was used to create a secondary focused screening plate to identify the optimal refolding conditions. DOE is a powerful tool to optimize multi-factorial systems via a rational choice of experimental conditions. The experimental design software suggests, based on the design chosen, a limited number of combinations of the variables reducing the number of conditions to test. In order to identify optimal conditions and improve the refolding yield, a fractional matrix D-Optimal design for 4 variable factors was applied, generating usually 26 conditions. The results from the focused screen were analyzed with the DOE software and the optimal refolding conditions were predicted.

Preparation of the focused refolding plate

The focused refolding plate was prepared with the AKTA explorer 100 chromatographic system following the same procedure as used for the generic master and screening plates. A master buffer plate with the conditions suggested by DOE was prepared. The denatured protein was added to a 96-well plate and the buffer preparations from the focused master buffer plate were added in 4 pulses as described for the primary generic screen. After dilution the plate was incubated, samples were filtered and protein concentrations were determined as described for the generic screen.

Refolding yield and quality analysis of the focused screen

The refolding yields and the quality of the refolded protein were analyzed by RP-HPLC as described above for the analysis of the generic screen. SDS–PAGE was systematically performed to control the purity of the product.

Scale-up refolding and purification of the refolded protein

To confirm the refolding conditions predicted in the focused screen, a medium-scale refolding was performed. Cell paste (10 g) was processed following the DOE optimized refolding protocol and the standard protocol in parallel when one was available. After refolding, the proteins were purified on a cation exchange column (SE-Hicap, XK 26/10 column) at a linear flow rate of 150 cm/h and eluted with a linear gradient of 0–1 M NaCl. The fractions containing the protein of interest were collected and dialyzed overnight against 0.1 M Tris–HCl pH 7.5. The concentration of the purified protein was determined by UV spectrometry using the molar extinction coefficient indicated for each protein. The amount of purified protein was determined and the refolding yield was calculated. The purity of the protein was verified by SDS–PAGE and the activity was tested in an *in vitro* assay.

Activity assays

Competition receptor binding assay

The activity of the refolded Met-SDF-1α was tested in a scintillation proximity assay (SPA) based on competition of radiolabeled ligand binding to its receptor. The assay was carried out with membranes from CHO transfected cells expressing the CXCR4 receptor (Millipore). [¹²⁵I] labeled CXCL12 was prepared by Perkin Elmer. Serial dilutions of unlabeled CXCL12, covering the range 10^{−5}–10^{−12} M, and containing 0.2 nM [¹²⁵I]-CXCL12 were prepared in binding buffer (50 mM Tris/HCl, pH 7.2, 1 mM CaCl₂, 5 mM MgCl₂ and 0.5% BSA). Wheat germ scintillation proximity assay (SPA) beads (GE Healthcare) were solubilized in PBS at 50 mg/ml, diluted in binding buffer to a concentration of 10 mg/ml, and membranes of the CHO cells were solubilized at 80 µg/ml in binding buffer. Equal volumes of membrane (25 µl) and SPA bead solutions (25 µl) were mixed before adding them to the assay. The final membrane concentration in the assay was 40 µg/ml and the final concentration of [¹²⁵I]-CXCL12 was 0.1 nM. The plates (Corning, 96-well, flat and clear bottom) were incubated under agitation for 4 h at room temperature. Radioactivity was counted with a beta counter for 1 min/well and the data were analyzed using GraphPad Prism software.

Chemotaxis assay

Chemotaxis assays were carried out in 96-well microplates (Neuro Probe ChemoTx) with L1.2/CXCR5 transfected cell. The cells were cultured in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 25 mM HEPES, 0.05 mM β-Mercaptoethanol and 0.8 mg/ml Geneticin G-418. The day before the assay, 5 mM *n*-butyric acid was added to the culture medium to induce expression of CXCR5. The cells were collected by centrifugation (600×g) and resuspended at a concentration of 0.85 × 10⁶/ml in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS) without phenol red. A dilution series of CXCL13 was prepared covering the range of 10^{−6}–10^{−12} M in RPMI medium without phenol red, and 30 µl were placed in the wells of the lower plate. A filter membrane (8 µm pore size) was placed over the plate. Twenty five microliters of the cell solution (2.5 × 10⁴ cells) was deposited on top of the membrane of each well. The chamber was incubated for 5 h at 37 °C under 5% CO₂. The cell suspension was then washed with PBS and the filter was

removed. The cells that migrated in the lower wells were transferred to a black plate using a 96 well funnel adaptor and frozen for at least 2 h at -80°C . The plate was thawed and 200 μl of a solution of CyQUANT dye/cell-lysis buffer mix (Molecular Probes), was added to each well. The fluorescence was measured using a Victor² Wallac plate reader and the data were analyzed using GraphPad Prism software.

C5a induced AKT phosphorylation assay

RAW264.7 (ATCC) is a macrophage type cell line on which C5a stimulation leads to a transient phosphorylation of AKT by phosphatidylinositol-3 kinase gamma (PI3K gamma). To perform the assay, 2×10^4 cells per well were grown overnight in medium containing serum. The cells were subsequently starved for 90–120 min after which they were stimulated with the different preparation of C5a at increasing concentrations as indicated in Fig. 8. The reaction was stopped after exactly 6 min by addition of 100 μl of 12.8% paraformaldehyde in PBS. The cells were permeabilized, and the endogenous peroxidase was inactivated by addition of 100 μl of 0.1% H_2O_2 for 20 min. To reduce non-specific antibody binding cells were incubated in 10% FCS for 60 min before incubation with an anti-p-AKT antibody over night (Ser 473 Akt AB 193H12 from Cell signaling diluted 1/500 in PBS). After four washes with PBS, the secondary antibody coupled to the Horseradish peroxidase (Dako, item P0448 diluted 1/500 in PBS) was incubated for 60 min. The cells were washed four times with PBS + 0.1% Triton X-100. For detection, 100 μl of chemoluminescent substrate (SuperSignal ELISA Pico from Thermo) was added in each well. Finally, the plates were analysed in a luminescence plate reader (Victor 3V from Perkin Elmer/Wallac) and the amount of AKT phosphorylation was determined.

GM-CSF dependent proliferation assay

The FDC-P1 cell line was obtained from ATCC and was propagated in growth medium supplemented with 10% fetal bovine serum and 2 ng/ml mouse GM-CSF in a 37°C incubator with 5% CO_2 as described by Dexter et al. [33]. Recombinant murine GM-CSF produced in *E. coli* was purchased from Peprotech (Ref. 315-05).

The proliferation assay was performed according to the protocol of Delamarter et al. with some modifications [31]. Carefully washed FDC-P1 cells were seeded at 4000 cell per well in a 96-well microtiter plate and incubated with a fourfold serial dilutions of recombinant mouse GM-CSF starting at 50 ng/ml for 48 h. Cell proliferation was measured by adding 1/10th volume of Alamar blue (Invitrogen) for 4 h and reading the fluorescence signal at 590 nm with excitation at 544 nm. EC_{50} values were determined using Prism V4.03 software (GraphPad) applying a non-linear regression curve fit to the dose-response curves obtained from the average of duplicate values.

MALDI-TOF-MS analysis

The samples were desalted by RP-HPLC into 0.1% TFA in water on a 3 cm $\times$ 4.6 mm Poros R/H column obtained from Perseptive Biosystems connected to an HPLC system from Agilent Technologies. The fraction containing the desalted protein was quantified by UV spectroscopy and 1 μg of protein was co-crystallized with 1 μl of sinapinic acid and analyzed in linear mode using an accelerating voltage of 25,000 V and a delay time of 200 ns. The instrument was calibrated with CalMix#3 (Insulin 5734 Da, Thioredoxin 11,674 Da and Apomyoglobin 16,952 Da). The matrix-assisted laser desorption/ionization reflection time-of-flight (MALDI-TOF) was performed on a Voyager DETM-PRO mass spectrometer (Applied Biosystems).

Results and discussion

A major hurdle for the production of recombinant proteins in bacteria is the necessity for refolding of proteins expressed in inclusion bodies. In particular, for proteins containing cysteine residues that need to be paired into correct disulfide bridges, the refolding can be a crucial step in the production process. Unfortunately, no universal buffer suitable for refolding of proteins has been identified, thus the refolding conditions must be identified for each individual protein. In order to facilitate the identification of suitable refolding conditions we have developed a two-step refolding screen. Before the refolding, the protein is partly purified on reversed phase chromatography to eliminate bacterial proteins and contaminants like endotoxins and DNA since those might affect the refolding efficiency. The purity of the proteins before refolding was estimated by SDS-PAGE to be between 90% and 95% (data not shown). The first step is a generic refolding screen composed of 96 different refolding conditions performed in a 96-well plate (Fig. 1). Additives that have been shown to affect the refolding efficiency of different proteins were included in the screen. Only one concentration of each additive was tested. The plate was prepared in an automated mode with an Akta Explorer 100 system, which reduced the risk of error in the preparations and assured a high reproducibility in plate preparation. In case an Akta is not available the plates could also be prepared in a manual way. The aim of this first screen was to determine if the protein was amenable to refolding and to identify the factors that had a major influence on the refolding. Based on the results of the primary screen a second focused screening plate containing a limited number of conditions was designed with the DOE software. A broad range of concentration was explored for each reagent with the aim to identify the optimal concentrations as well as interactions between the various additives. Based on the results of the focused screen the DOE software is able to predict the composition of the optimal refolding buffer. In most available refolding screens the composition of the denaturing solution is not taken into account. Since the ratio of reduced and oxidized glutathione is frequently a critical factor, the concentration of DTT in the denaturing solution could influence this balance; therefore we included this parameter in the second focused screen.

Determining the refolding yield and analyzing the quality of the refolded protein is not obvious, nevertheless it is the most important readout to estimate the refolding efficiency. In most of the commercial screens available, this step is not defined and it is up to the customer to identify and apply a suitable technique. Several techniques has been used, such as light scattering, SDS-PAGE, size exclusion chromatography (SEC) and RP-HPLC chromatography, however some of these are time consuming and require significant investment. In this study analytical RP-HPLC was used as a generic method for estimation of the refolding efficiency. The method is based on the differences in hydrophobicity between denatured and folded protein. During the folding process non-polar side chains are buried inside the structure making the protein less hydrophobic which results in a weaker interaction with the RP resin and the protein is easily eluted from the column. The unfolded form, exposing larger hydrophobic areas, is better retained on the resin and elutes later in the elution gradient (Fig. 3). This is a powerful method for assessing protein folding, it is easy to set up, consumes small amounts of material, and has a high sensitivity and reproducibility. Furthermore, elution fractions can be directly analyzed on SDS-PAGE for determination of purity and by MS-MALDI-TOF to verify that disulfide bridges have been correctly formed.

The protein used for setting up of the screen was IL-17F, a homodimer of 30 kDa composed of two 134 amino acid polypeptides folded together with two intramolecular and two intermolecular

disulfide bridges. Involving both intermolecular and intramolecular disulfide bridge formation, the structure of the molecule is relatively complex making refolding a challenge. IL-17F was enriched from the inclusion bodies extract by RP chromatography. The protein eluted from the RP column was lyophilized and solubilized in 6 M guanidine HCl in 0.1 M Tris pH 7.5, 2 mM DTT at a concentration of 4 mg/ml. The purity was estimated to 91% by SDS–PAGE (data not shown). The protein was submitted to the refolding screen, after which the amount of soluble protein was determined (Fig. 2). The results showed that the pH was one of the most critical factors, and that the recovery of soluble protein gradually increased with increasing pH. Low amount of soluble protein was obtained at pH 6.5, higher amounts at pH 7.5 and 8.5, whereas the highest amounts were found in conditions at pH 9.5, above the isoelectric point of the protein which is predicted to be 8.82. The screen also demonstrated that the redox condition was critical for correct disulfide bridge formation. At pH 9.5 refolding conditions with GSH/GSSG at a ratio of 3 mM/0.3 mM (Fig. 2, conditions G) gave significantly higher yields of soluble protein compared to the same conditions with a ratio of 0.3 mM/3 mM (Fig. 2, conditions H). Furthermore, L-arginine was identified to promote refolding of this protein, and a favorable effect of NDSB 256 was also detected. The soluble fractions from the 20 conditions with the highest yields were analyzed on analytical reversed phase chromatography. The column was calibrated with folded native IL-17F (retention time 15.4 min) and the protein denatured in 6 M guanidine with 10 mM DTT (retention time 15.7 min). A difference of 0.3 ml in elution volume was seen between the two protein forms, demonstrating that the column has the resolution to separate the folded from the denatured form of the protein (Fig. 3). Of the 20 samples analyzed, several showed a substantial portion of the soluble protein coeluting with the correctly folded protein peak, and the most promising candidates have been overlaid with the standards (Fig. 3). The refolding condition G7 demonstrates

that relying only on the amount of soluble protein can be misleading (Figs. 2 and 3). This condition gave the highest amount of soluble protein, however, the RP analysis shows that almost the entire amount coeluted with the peak corresponding to unfolded protein. The results also demonstrated the impact of glutathione on the refolding efficiency. Although, correctly refolded protein was recovered at a low amount under oxidizing condition at pH 9.5 (conditions G), changing the glutathione ratio to reducing conditions significantly increased the yield of refolded protein (conditions H). NDSB 256 slightly increased the refolding efficiency, however, due to the high cost of the reagent and the marginal effect it was not included in the focused secondary screen. In addition to the RP–HPLC analysis, the quality of the soluble fractions was analyzed on non-reducing SDS–PAGE. The results correlated well with the RP analysis, all the samples containing refolded protein on RP showed a band at a molecular weight of 30 kDa corresponding to the homodimer (data not shown).

Based on the results from the generic screen, three important factors for the refolding of IL-17F were identified: L-arginine, ratio of reduced and oxidized glutathione and the pH of the refolding buffer. In the screen only one concentration of each additive was tested. In order to identify the optimal concentrations of the additives, the design of the second focused screen was assisted by DOE. The redox environment appeared to be important for the refolding efficiency. Thus, in addition to the three factors identified by the primary screen, the influence of DTT in the denaturing buffer was also included. Twenty six conditions, combining different molarities for each additive, were proposed by the DOE software following a full fractional matrix D-Optimal design (Table 1). The focused refolding plate was prepared, the refolding performed, the soluble fractions recovered and the samples analyzed on RP chromatography and non-reducing SDS–PAGE as for the generic screen (Fig. 4). Condition 7 was significantly better with regards

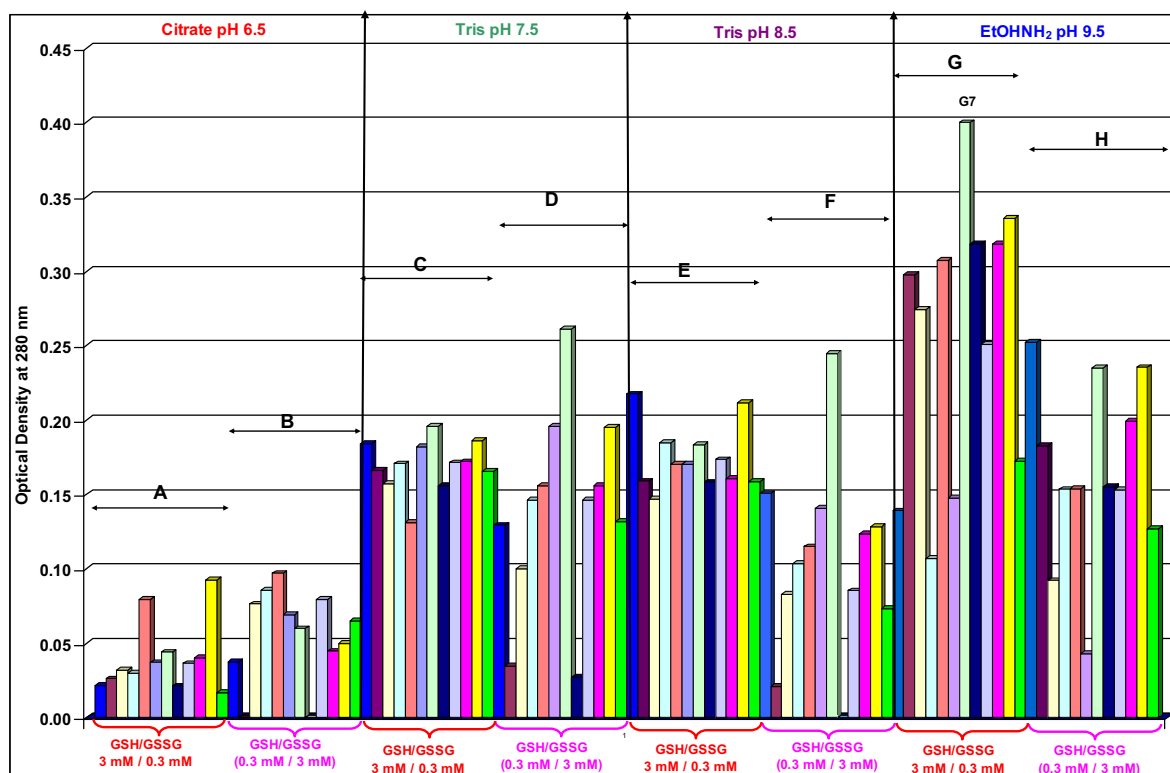


Fig. 2. Yields from the primary generic refolding screen for Met-IL-17F. After the refolding the samples were filtered at 0.45 μ m on a MultiScreen Deep Well Solvintert hydrophilic low binding PTFE plate and the optical density was measured between 220 and 320 nm. The absorption at 280 nm was used to estimate the protein amount in the soluble fraction.

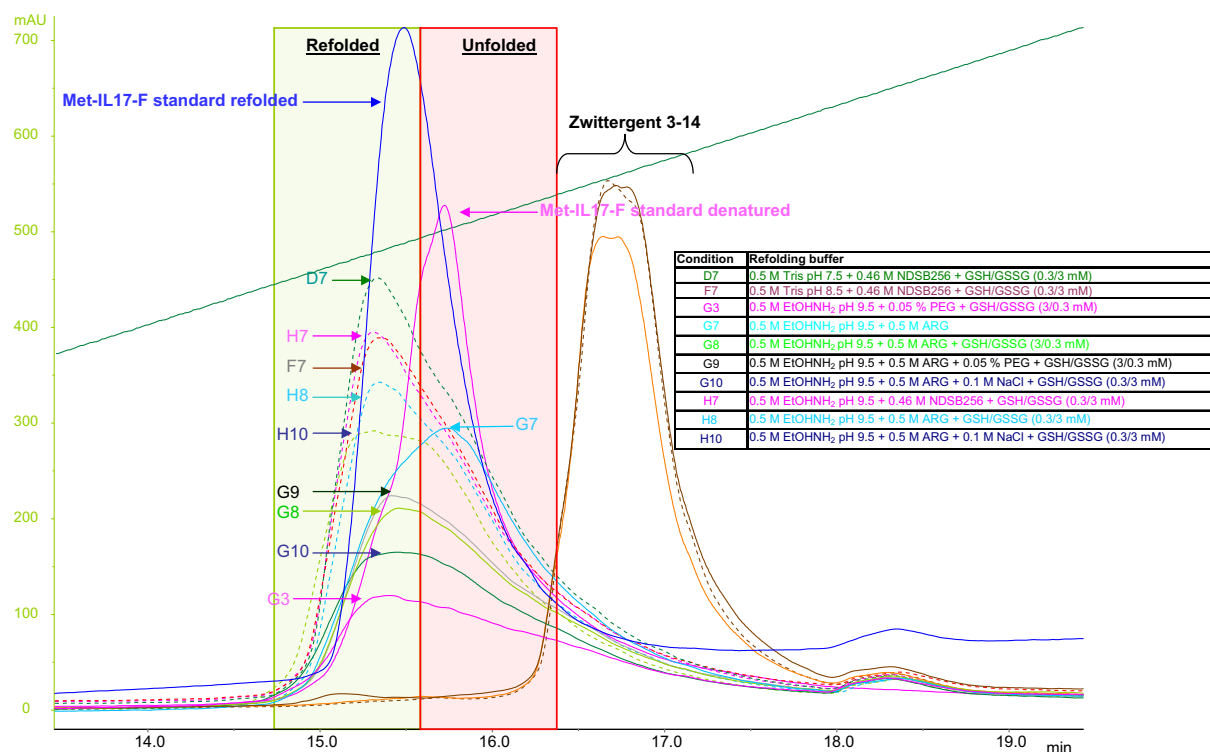


Fig. 3. Refolding quality of Met-IL-17F analyzed by RP-HPLC. The refolding conditions that gave highest refolding yields were analyzed on RP-HPLC to determine the refolding quality. The column was calibrated with folded (retention time 15.4 min) and unfolded (retention time 15.7 min) Met-IL-17F, and the elution profiles at 214 nm of the refolded samples were compared to the retention times and elution profiles of the reference samples. Zwittergent 3-14 included in some of the refolding conditions eluted at the end of the elution gradient as indicated in the figure.

Table 1
Matrix for the second focused screen for Met-IL-17F. In order to optimize the refolding conditions for the crucial factors identified in the first screen, a second focused refolding plate consisting of 26 different conditions were generated by the DOE software on a D-optimal matrix design. The refolding yield and the protein quality, assessed as the percentage of dimer on non-reducing SDS-PAGE in the RP-HPLC purified refolded protein samples, were used to identify the best refolding conditions.

Design of experiment number	(EtOHNH ₂) in mM	(GSH) in mM	(GSSG) in mM	(ARG) in mM	(DTT) in mM	Refolding yield assessed by RP-HPLC (%)	% of dimer assessed by densitometer on SDS-PAGE
1	50	3	0.1	0	2	3	32
2	275	1.55	3	250	1	4	14
3	275	3	1.55	0	1	10	41
4	500	3	0.1	500	1	14	22
5	500	0.1	0.1	0	2	7	39
6	50	0.1	0.1	0	0	7	19
7	50	0.1	0.1	500	2	31	34
8	50	0.1	3	500	0	7	18
9	500	3	0.1	0	0	11	14
10	50	3	3	0	0	6	33
11	50	3	3	500	2	19	10
12	50	1.55	1.55	500	2	14	14
13	500	0.1	1.55	0	1	4	18
14	275	0.1	1.55	250	2	11	9
15	50	1.55	0.1	250	1	6	21
16	500	0.1	3	500	2	14	11
17	50	3	0.1	500	0	5	42
18	500	0.1	0.1	500	0	12	12
19	275	3	0.1	500	2	8	16
20	275	1.55	1.55	500	0	14	8
21	500	3	3	500	0	13	12
22	500	0.1	3	0	0	4	10
23	500	1.55	0.1	500	2	18	10
24	500	3	3	0	2	10	14
25	500	3	1.55	250	2	15	10
26	50	0.1	3	0	2	7	23

to both the refolding yield and the quality of the refolded protein (Table 1 and Fig. 4). On RP-HPLC 31% of the protein coeluted with the peak corresponding to the folded protein, which was the

highest yield obtained (data not shown). The percentage of dimer on SDS-PAGE determined by densitometry scanning was 34%. Thus, the best refolding yield in the focused screen was obtained

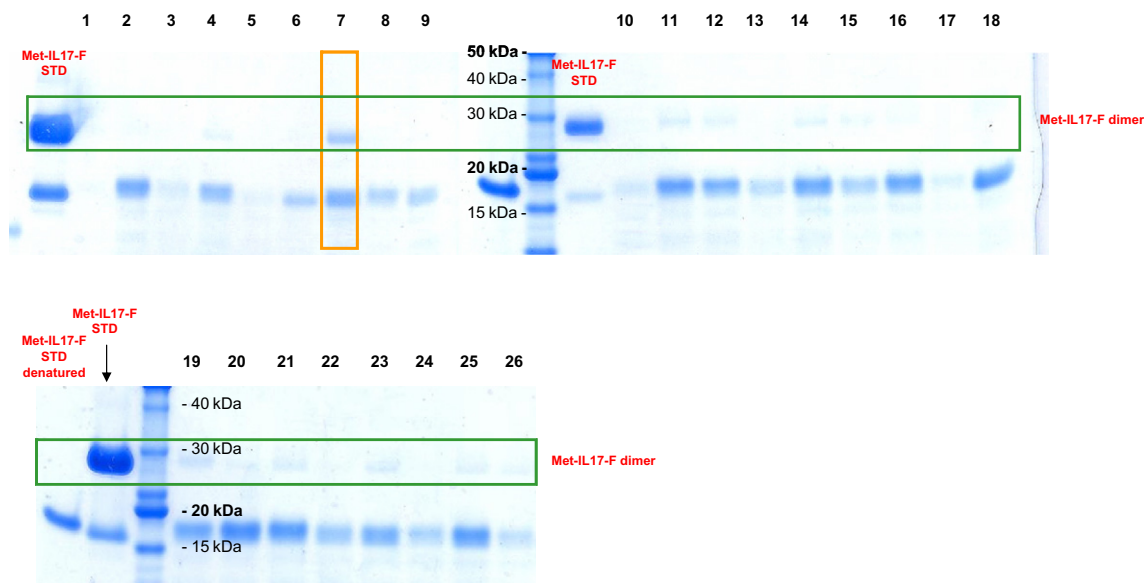


Fig. 4. Analysis of refolding quality of Met-IL-17F by SDS-PAGE. The soluble fraction from each refolding condition was purified on RP-HPLC. The fraction containing the refolded protein was lyophilized, resolubilized in 0.5 M Tris-HCl, pH 7.5 and analyzed on non-reducing SDS-PAGE, except the denatured control which was in reducing conditions (8 M urea, 10 mM DTT, 0.1 M Tris, pH 7.5).

with 2 mM DTT in the denaturation buffer and with 50 mM ethanolamine at pH 9.5, 0.5 M L-arginine and GSH/GSSG (0.1 mM/0.1 mM) as renaturation condition.

In order to mathematically verify and refine the concentrations of each additive used to identify the optimal refolding conditions, the data presented in Table 1 were analyzed by the DOE software. The statistical analysis showed that the model selected fit well with the response for both refolding yield and dimer formation assessed by SDS-PAGE [$R^2_{\text{refolding yield}} = 0.945$; $R^2_{\% \text{ of dimer}} = 0.922$]. Fig. 5 shows the predicted responses on yield and dimer formation as a result of changes in the concentration of the various factors. Increasing the DTT concentration in the denatured sample as well as the L-arginine concentration in the refolding buffer is calculated to significantly improve the refolding yield, whereas dimer formation resulted in bell shaped dependent curves for both factors. Reducing the buffer concentration and the reduced and oxidized glutathione had a positive impact on the refolding yield. The yield of dimers was also positively affected by decreased buffer and oxidized glutathione concentrations, whereas reduced glutathione showed a bell shaped concentration dependence. In summary, apart from pH identified in the first screen, the two main factors having a significant impact on the refolding yield were L-arginine ($p < 0.01$) and DTT ($p = 0.05$), whereas the concentration of ethanolamine had much less of an effect. Furthermore, a major effect on the yield was attributed to the ratio of reduced and oxidized glutathione ($p = 0.044$). Based on the predictions, the optimal refolding conditions were suggested to be with 1.5 mM DTT in the denatured sample and a refolding solution composed of 50 mM ethanolamine, pH 9.5, 0.5 M L-arginine, GSH/GSSG (0.2 mM/0.2 mM). This refolding condition was a compromise between the predicted conditions that gave the highest refolding yield and the highest formation of dimer (Fig. 5). For this condition the software predicted a refolding yield of 25% with 37% dimer, or overall yield of 10% properly refolded dimers. The identified refolding conditions were validated at medium scale. The refolding process was identical to the process used in the screening format, only the volumes were scaled up by a factor of 500. In addition, at the end of the refolding the protein was purified on cation exchange chromatography to isolate the refolded dimer. The optimized refolding process was compared to the standard procedure

previously used. The refolding yield was improved from 11% to 20% (Fig. 6), close to the prediction of 25%. Compared to the standard process the yield of dimers after cation exchange chromatography was improved from 5% to 11%. Again close to the predicted yield of 10%. The purity was 92% as determined from non-reducing SDS-PAGE (Fig. 7A, lanes 1–2) with the expected band around 30 kDa corresponding to the homodimer. A weak band seen at 15 kDa corresponds to the monomer, which in solution presumably forms a non-disulfide linked dimer.

To validate the screen as a generic approach for identification of suitable refolding conditions, the approach was tested on 4 additional proteins. First, the screen was applied to the small chemokine SDF-1 α /CXCL12 belonging to the CXC chemokine family. The protein was denatured in 0.1 M Tris/HCl pH 8, 6 M guanidine, 2 mM DTT. The denatured protein was submitted to the 96-well primary refolding screen and the samples were analyzed as described for IL-17F. Sucrose and the ratio of reduced/oxidized glutathione showed a clear positive impact on the refolding, L-arginine had a slight effect, whereas the buffer pH, detergent and salt had no significant influence. Based on the results from the primary screen, the secondary screening was focused on sucrose concentration, glutathione ratio, L-arginine concentration and DTT concentration in the denaturing solution. 21 conditions generated with the DOE program was performed in 0.1 M Tris buffer at pH 7.5, which showed to be the most suitable buffer in the generic screen. The result of the focused screen was analyzed with the DOE program to identify the optimal refolding conditions. The results showed that addition of 0.3 M sucrose significantly increased the refolding yield ($p = 0.06$) as well as removing DTT from the denatured sample ($p = 0.001$). Moreover, an equal ratio of reduced and oxidized glutathione (0.15/0.15 mM) increased the yield. The suggested condition was tested at medium-scale refolding. After refolding, SDF-1 α was purified on a cation exchange column and the purity was estimated to be over 95% by SDS-PAGE (Fig. 7A, lanes 4–5). Proudfoot et al. [34] developed a generic refolding protocol for chemokines and demonstrated that refolding of chemokines is strongly influenced by the ratio of GSH/GSSG in the refolding buffer and the concentration of DTT in the denaturing buffer. The proposed refolding buffer was composed of 20 mM Tris-HCl buffer pH 8 and GSH/GSSG (0.1 mM/0.01 mM). This

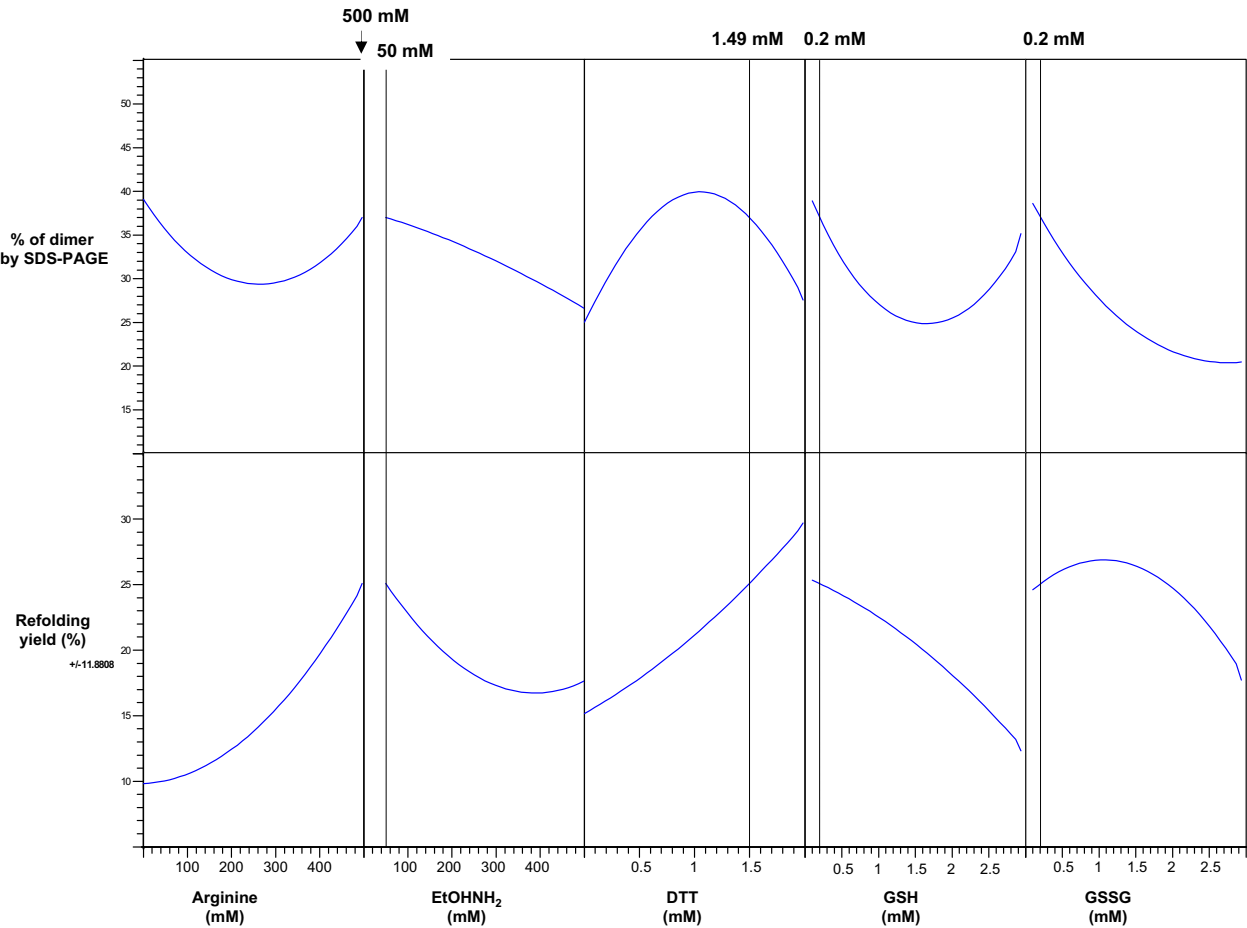


Fig. 5. Predictions of optimal refolding conditions for Met-IL-17F. Based on the results obtained from the focused secondary screen the DOE software predicted the optimal concentrations for the critical additives for refolding of Met-IL-17F based on refolding yield and dimer formation. The vertical dotted lines represent the optimal concentrations for each additive to obtain the highest yield (bottom row) with the best protein quality, dimers (top row). The predicted optimal concentrations of each additive are indicated at the top of the figure over the dotted lines.

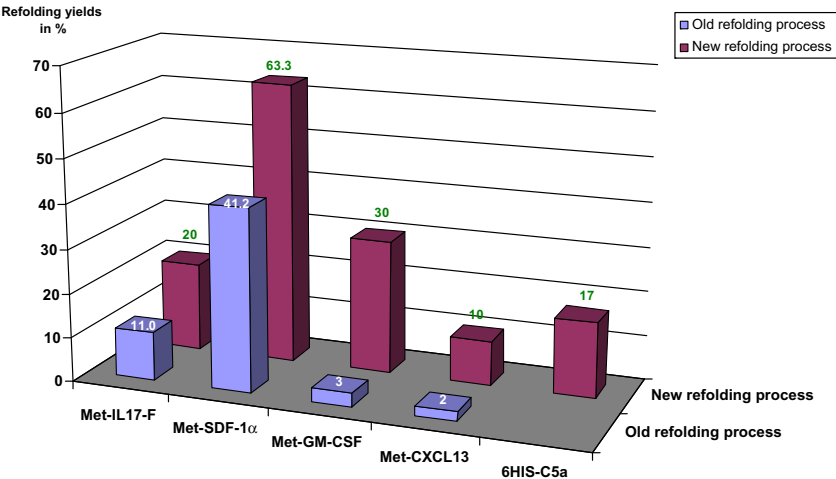


Fig. 6. Refolding yields in medium-scale productions for the 5 proteins. A summary of the final refolding yields after purification for the five proteins with the optimized refolding processes, compared to the standard protocols, when data was available.

protocol has successfully been used to refold SDF-1 α . Following that procedure a refolding yield of 41% was obtained. With the newly identified refolding conditions, the refolding yield increased with approximately 53% (Fig. 6). In order to verify that the protein was correctly refolded and that disulfide bridges had been formed

the protein was analyzed by MALDI-TOF-MS. The mass peak at 8091 Da matched perfectly the theoretical mass indicating that 4 hydrogen atoms were lost due to disulfide bridge formation (Fig. 7B). The refolded protein was further analyzed in a CXCR4 receptor binding assay. No significant differences in the EC₅₀ value

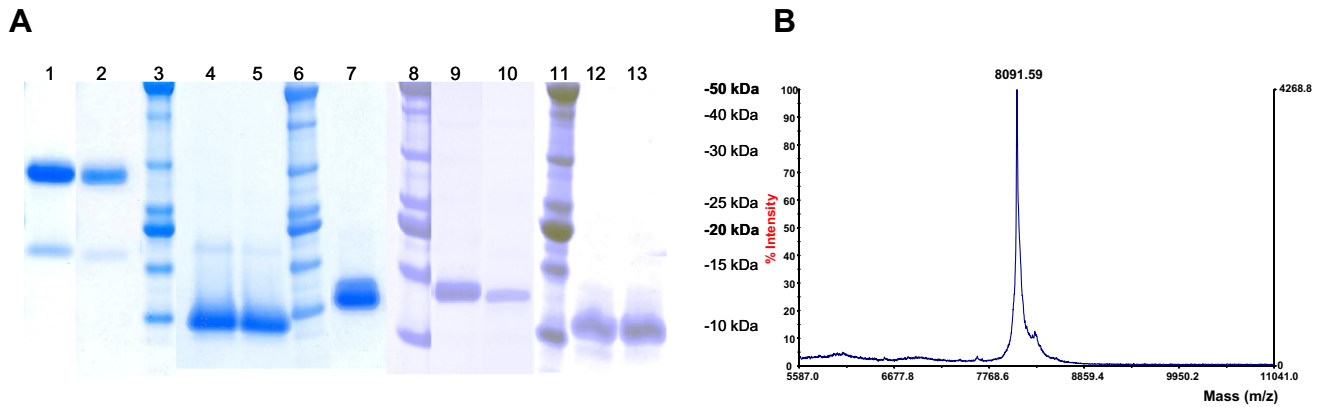


Fig. 7. Panel A. Protein quality analysis on SDS–PAGE. Analysis of the refolded and purified proteins from medium-scale productions on non-reducing SDS–PAGE. Proteins refolded with the newly identified optimized refolding protocols compared to the standard protocols. Lane 1: Met-IL-17F, optimized refolding protocol. Lane 2: Met-IL-17F, standard refolding protocol. Lane 3: Benchmark protein ladder. Lane 4: Met-SDF-1 α , optimized refolding protocol. Lane 5: Met-SDF-1 α , standard refolding protocol. Lane 6: Benchmark protein ladder. Lane 7: 6HIS-C5a, optimized refolding protocol. Lane 8: Benchmark protein ladder. Lane 9: GM-CSF, optimized refolding protocol. Lane 10: GM-CSF, standard refolding protocol. Lane 11: Benchmark protein ladder. Lane 12: Met-CXCL13, standard refolding protocol. Lane 13: Met-CXCL13, optimized refolding protocol. Panel B. Analysis of SDF-1 α by MALDI-TOF-MS. The refolded and purified protein was analyzed on MS. A mass peak at 8091.59 Da corresponding to the refolded protein was detected.

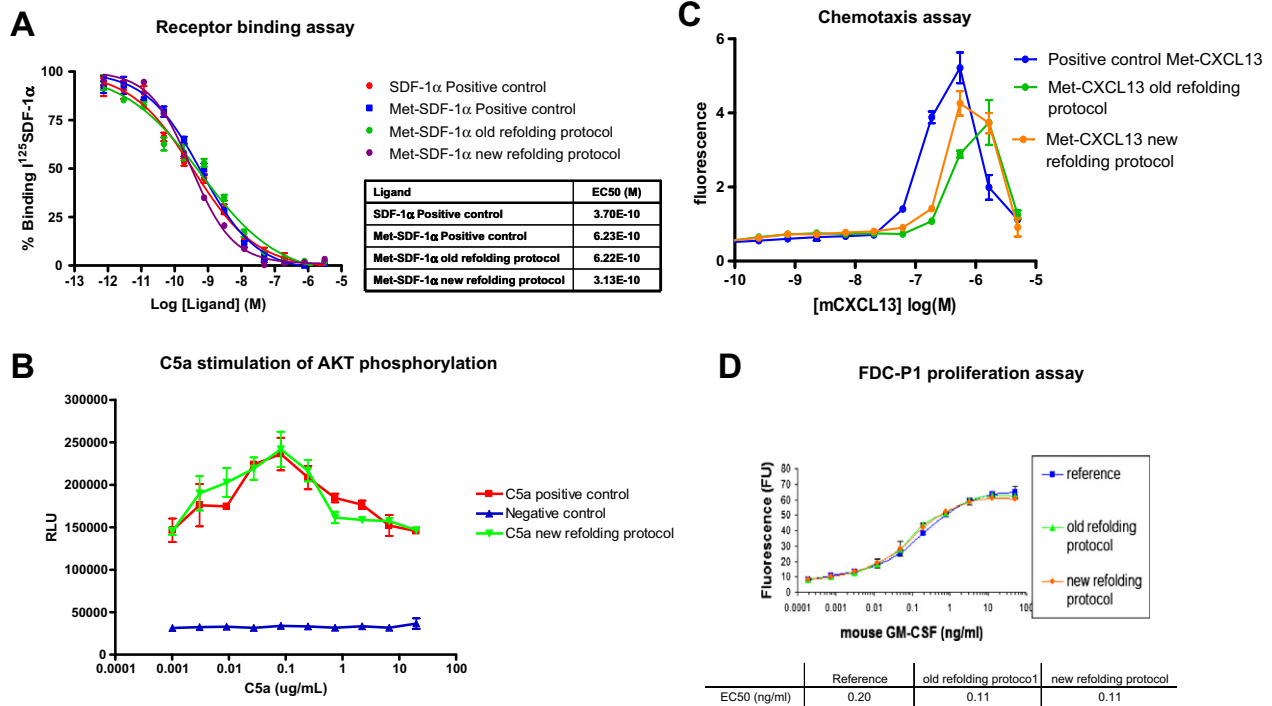


Fig. 8. Protein activity assays. The refolded and purified proteins were tested in *in vitro* activity assays as described in the Methods section. The activities of the refolded proteins produced with the optimized protocol were compared to proteins prepared with standard protocols and reference batches as indicated in the figure panels. (A) Met-SDF-1 α proteins tested in a SPA based CXCR4 receptor binding assay on isolated cell membranes. (B) 6HIS-C5a proteins tested in a C5a stimulated AKT phosphorylation assay. (C) Met-CXCL13 proteins tested in a chemotaxis assay on L1.2 murine cells expressing CXCR5. (D) GM-CSF tested in a GM-CSF dependent FDC-P1 cell proliferation assay.

was found compared to SDF-1 α produced with the standard refolding protocol or to a reference standard, demonstrating that the protein was fully active (Fig. 8A).

For further validation the refolding screen was applied to the small complement protein C5a. The critical additives identified to increase the refolding efficiency were polyethylene glycol ($p = 0.05$) and the concentration of reduced glutathione ($p = 0.04$). To optimize the refolding a focused screen was carried-out. The concentration of DTT in the denatured extract as well as different ratios of reduced and oxidized glutathione, and several concentrations of polyethylene glycol 3300 in the refolding buffer were

tested. The focused screen demonstrated that concentration of reduced glutathione was the key factor to improve the refolding of the C5a protein. Thus, the more we increased the concentration of reduced glutathione, the more we increased the refolding yield. In RP-HPLC chromatography folded and denatured forms of this protein were very well separated, which facilitated the determination of the refolding efficiency for the different conditions. From the screening results the DOE program suggested a refolding buffer composed of 0.1 M Tris–HCl pH 7.5, 0.1% PEG 3300, 2 mM reduced glutathione, 0.2 mM oxidized glutathione and without DTT in the denatured sample. When this condition was tested in a

medium-scale refolding experiment, an overall yield of 17% with a purity of over 95% on SDS–PAGE was reached after RP purification (Figs. 6 and 7A lane 7). To ensure that the protein was correctly folded, the capacity of C5a to induce AKT phosphorylation by PI3K gamma was tested in an *in vitro* activity assay. The 6HIS–C5a produced in-house was found to be as active as a commercial reference batch (Fig. 8B). The refolding conditions identified by our screening approach are similar to those described by Fleming et al. [29]. An advantage in our protocol is that the refolding is performed by a limited dilution, whereas in the Fleming protocol dialysis against a 1000-fold volume was applied, which makes handling of the sample more complicated, particularly at a scale-up level.

The chemokine CXCL13, which had proven to be challenging to refold with a yield of only approximately 2%, was submitted to the screening procedure. The primary screen showed that pH had no real influence on the refolding as long as it was higher than 7 to allow formation of disulfide bridges. The first screening demonstrated that NDSB 256 as well as the ratio of glutathione had a positive influence on the refolding yield. A focused screen with different ratios of glutathione and varying concentrations of NDSB 256 in the refolding solution was carried-out. Different concentrations of DTT in the denaturing buffer were also tested. The results from the focused screen showed that an excess of GSSG ($p < 0.06$) compared to GSH was the key for successful refolding of CXCL13 in order to establish correct disulfide bridges. A new refolding buffer composed of 0.1 M Tris pH 7.5, 0.46 M NDSB 256, 0.5 mM GSH, and 3.3 mM GSSG was identified. A low concentration (0.5 mM) of DTT was maintained in the denaturation buffer to preserve a reducing environment before renaturation without affecting the refolding yield. In a medium-scale refolding test with the optimized refolding conditions the yield increased to 10% with a purity of over 95% on SDS–PAGE (Figs. 6 and 7 lanes 12–13). The activity of the protein was tested and compared to a batch produced with the standard refolding conditions and a positive benchmark in a chemotaxis assay. No significant difference in activity was detected between the three protein batches (Fig. 8C).

The last protein tested in the refolding screen was GM-CSF. Critical factors identified in the primary screen were the pH and the ratio of GSH/GSSG. Precipitation occurred at pH 6.5 and the refolding yield increased significantly at pH 9.5. Addition of NDSB 256 had a positive effect on the refolding yield by reducing precipitation. All the other additives like salt, L-arginine, Zwittergent did not improve the refolding yield. Based on these results, the optimal concentrations and composition of the refolding buffer was identified in the focused screen. The results showed that the concentration of GSSG was the most important factor for the refolding success. Increasing the concentration of GSSG up to 1.5 mM significantly increased the refolding yield ($p = 0.08$), whereas the concentration of DTT in the denatured sample had no influence ($p = 0.9$). The suggested optimal refolding condition was 50 mM ethanolamine pH 9.5, 0.2 M NDSB, GSH/GSSG (0.15 mM/1.5 mM). In a medium-scale refolding test using this refolding condition, the refolding yield rose from 3% to 30% with a purity of over 95% (Figs. 6 and 7, lanes 9–10). The activity of the Met-GM-CSF produced with the optimized protocol was not significantly different from the protein produced with the standard refolding conditions and the commercial reference protein (Fig. 8D).

Conclusions

We have developed a two-step protein refolding process, composed of a primary generic 96-well based screening assay for the identification of crucial factors affecting protein refolding, followed by a protein specific focused secondary screen supported by DOE

for the identification of the optimal refolding conditions. DOE allows to not only identify the critical additives, but also to determine the optimal concentrations. In this study we used the Cornerstone DOE software from P&P Informations technologie that is able to generate many different kinds of experimental designs, including the D-optimal design. However, in principal any DOE program could be used to support the screening process. Furthermore, we have included a RP-HPLC analysis step to determine the refolding efficiency, which is frequently based only on the amount of soluble protein recovered, which is not a very accurate estimation of correctly refolded protein. The screening plates are produced in the laboratory from ordinary chemicals and unlimited numbers of plates can be produced at very reasonable costs. Since the plates are produced in the laboratory, the focused screen is tailor made to the individual proteins which makes the screen very flexible and delivers more accurate and relevant results. This refolding approach has been applied to five proteins of very different structure. The identified refolding conditions were validated in medium-scale refolding tests, and for all of the tested proteins we were able to increase the refolding efficiency compared to established protocols. The whole screening approach requires less than 40 mg of denatured protein, and gives reliable, reproducible results in a short time. The complete procedure from defining the critical parameters up to determining the optimal refolding conditions can be performed in less than 2 weeks.

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