An automated in vitro protein folding screen applied to a human dynactin subunit

CHRISTOPH SCHEICH,^{1,2} FRANK H. NIESEN,^{1,3} ROBERT SECKLER,⁴ AND KONRAD BÜSSOW^{1,2}

¹Proteinstrukturfabrik, Heubnerweg 6, 14059 Berlin, Germany

²Max Planck Institut fuer Molekulare Genetik, Ihnestrasse 73, 14195 Berlin, Germany

³Institut fuer Medizinische Physik und Biophysik, Charité Universitaetsmedizin Berlin, 10117 Berlin, Germany

⁴Institut für Biochemie und Biologie, Universitaet Potsdam, 14476 Golm, Germany

(RECEIVED September 17, 2003; FINAL REVISION October 21, 2003; ACCEPTED October 21, 2003)

Abstract

The preparation of proteins for structural and functional analysis using the *Escherichia coli* expression system is often hampered by the formation of insoluble intracellular protein aggregates (inclusion bodies). Transferring those proteins into their native states by in vitro protein folding requires screening for the best buffer conditions and suitable additives. However, it is difficult to assess the success of such a screen if no biological assay is available. We established a fully automated folding screen and a system to detect folded protein that is based on analytical hydrophobic interaction chromatography and tryptophan fluorescence spectroscopy. The system was evaluated with two model enzymes (carbonic anhydrase II and malate dehydrogenase), and was successfully applied to the folding of the p22 subunit of human dynactin, which is expressed in inclusion bodies in *E. coli*. The described screen allows for high-throughput folding analysis of inclusion body proteins for structural and functional analyses.

Keywords: Folding screen; refolding; inclusion bodies; hydrophobic interaction chromatography; tryptophan fluorescence spectroscopy; human dynactin; structural genomics

Upon overexpression in *Escherichia coli*, a large proportion of proteins accumulates in insoluble, intracellular aggregates. These inclusion bodies contain high amounts of the target protein in relatively high purity. The structures of inclusion body proteins differ from their respective native conformations, for example, an increased level of nonnative β -sheets appears to be common among them (Fink 1998).

Inclusion body protein, solubilized by denaturants or detergents, can be folded to the native conformation by transfer into an appropriate buffer, using dilution or dialysis. This process is in some cases prone to high losses due to misfolding and aggregation, but may be shifted to higher yields by "folding helpers" (e.g., polyethylene glycol, glycerol, detergents, arginine, etc.). Many other factors including pH, protein concentration, or the redox milieu can influence the success of folding of a particular protein. Protein folding of denaturant- or detergent-solubilized proteins has been extensively reviewed (Guisse et al. 1996; Rudolph and Lilie 1996; Lilie et al. 1998; Misawa and Kumagai 1999; Clark 2001; Middelberg 2002).

Protein folding has also been achieved by binding the protein to a chromatographic resin in the unfolded state and subsequent washing with an appropriate buffer that contains no denaturant. Affinity resins like NiNTA sepharose (Zahn et al. 1997; Rehm et al. 2001) or heparin sepharose (Stempfer et al. 1996) have been used for poly-His-tagged

Reprint requests to: Christoph Scheich, Proteinstrukturfabrik, Heubnerweg 6, Berlin 14059, Germany; e-mail: scheich@molgen.mpg.de; fax: 49-30-32639-2833.

Abbreviations: CAB, bovine carbonic anhydrase II (from bovine erythrocytes); CD, circular dichroism; CTAB, hexadecyltrimethylammonium bromide; DLS, dynamic light scattering; DTT, dithiothreitol; FTIR, Fourier-transform infrared; GdmHCl, guanidine hydrochloride; GSH, reduced glutathione, GSSG, oxidized glutathione; HIC, hydrophobic interaction chromatography; IPTG, isopropyl β -D-1-thiogalactopyranoside; MDH, malate dehydrogenase (from pig heart mitochondria); TEV, tobacco etch virus.

Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.03304604>.

or poly-Arg-tagged proteins, respectively. Compared to folding by dialysis or rapid dilution, this method has two advantages. Aggregation due to intermolecular interaction of partly folded species is prevented, and the protein is obtained in higher concentration after folding. However, an interference of the chromatographic support with the folding protein molecule can be detrimental, causing precipitation of the protein on the matrix.

A protein folding screen for identification of optimal folding conditions using rapid dilution or dialysis has been developed by Gouaux and colleagues (Chen and Gouaux 1997; Armstrong et al. 1999). Related screens have been used by others (Heiring and Muller 2001; Tobbell et al. 2002). This so-called fractional factorial screen is also commercially available (FoldIt Screen, Hampton Research) and consists of an initial screen of 16 conditions, in which 12 parameters (various additives, pH, protein concentration) are altered. Each parameter appears in eight of the 16 conditions. Therefore, a couple of parameters are varied and tested at the same time, instead of checking each parameter independently. Thus, it is an approach to screen a high number of parameters with a relatively small number of folding buffers.

When screening for the best conditions for protein folding, it is difficult to determine the yield of native protein obtained if no biological assay is available. Centrifugation or filtration can remove precipitates, but solubility cannot be used as a stringent criterion, because soluble microaggregates and partially folded intermediates are not distinguished from the natively folded protein. To prevent aggregation, low protein concentrations are commonly used in folding experiments. Therefore, sensitive methods are required for detection. Analytical gel filtration can be used (Chen and Gouaux 1997). However, protein concentrations are often too low to detect folded protein. In addition, the method is time-consuming. Another method applied is limited proteolysis (Heiring and Muller 2001). Partially folded intermediates are assumed to be more susceptible to subtilisin-induced proteolysis than native protein. Although this approach is very sensitive and does not require high sample purity, there are some disadvantages. Range-finding experiments for the protease concentration may be necessary. Additionally, false positives or false negatives can occur because soluble nonnative microaggregates might be equally or even less susceptible to proteolysis than native protein, thus leading to a misinterpretation of the results.

In this work, we describe a folding screen that is performed automatically using a pipetting robot. In contrast to a fractional factorial screen we chose a screening-strategy in which only one parameter is changed at a time. The tested conditions comprise folding by rapid dilution and, in the case of His-tagged proteins, folding on metal chelate resins.

Tryptophan fluorescence spectroscopy was evaluated as a monitor to detect folded protein, as unfolded conformations,

folding intermediates, and native proteins are distinguishable in their respective spectra (Royer 1995). The wavelength of the fluorescence intensity maximum is shifted to smaller values (blue-shift) upon folding to the native state from the denaturant-unfolded protein. We tested whether the occurrence of a blue-shift is an indicator for folded protein in a folding screen.

Tryptophan residues are commonly more solvent exposed in unfolded or partially folded proteins than in native proteins. Solvent exposure usually causes quenching of tryptophan fluorescence. Therefore, we assumed that high fluorescence intensity is an indicator for folded protein. However, this assumption does not hold for all proteins. A few exceptions are known (e.g., γ F-crystallin; Das and Liang 1998) where the tryptophan fluorescence is strongly quenched in the native conformation by histidines, disulfide bonds, or chromophores. Hence, fluorescence intensity should only be used in conjunction with other folding monitors.

For some proteins, the evaluation of a folding screen with tryptophan fluorescence might be difficult due to low signal intensity; other proteins do not contain any tryptophans. Therefore, we tried to find an alternative method and tested whether hydrophobic interaction chromatography (HIC) can be used as a monitor to detect folded protein. During HIC, proteins are separated due to differences of surface hydrophobicity. Therefore, HIC should discriminate the compact native protein from partially folded or misfolded species that usually expose more hydrophobic patches. Soluble aggregates, which are assumed to form via interaction of hydrophobic patches between folding intermediates (Fink 1998), should also be distinguished.

An evaluation of the efficiency of the automated folding screen with the described biophysical analysis system was performed by refolding two denatured model enzymes, bovine erythrocyte carbonic anhydrase II (CAB), and pig heart mitochondrial malate dehydrogenase (MDH), and correlating the obtained data with enzymatic activity. In vitro refolding of CAB is strongly hampered by aggregation. CAB has therefore been used as a model to study aggregation processes during refolding (Cleland and Wang 1990). MDH was shown to be a substrate of the GroEL/GroES folding system during in vitro refolding (Weber et al. 1998). In this study, the chaperones enhanced MDH refolding efficiency by more than a magnitude compared to refolding without GroEL/GroES.

Furthermore, we tested whether the developed system is suitable to determine conditions to fold the p22 subunit of human dynactin that is expressed as inclusion bodies in *E. coli*. Dynactin is a macromolecular complex that is an activator for cytoplasmic dynein-mediated transport (Gill et al. 1991). The first report on the smallest subunit, p22 (21 kD), was by Holzbaur and coworkers (Karki et al. 1998). In this study, the authors noted a novel localization for cyto-

plasmic dynein and dynactin at the cleavage furrow and the midbody of dividing cells. So far, no structural information of the dynactin subunits is known. For the p22 subunit, the closest structural homolog is the β -domain of streptokinase, with a sequence identity of 25%, covering 53% of the sequence of p22 dynactin. Therefore, we tried to obtain a preparation of pure and native p22 dynactin for structural analysis.

Results

The folding screen consists of 20 conditions for rapid dilution with seven parameters (pH and additives; Table 1). Several small protein aliquots were added to each folding buffer in 2.5-h intervals to achieve low concentrations of aggregation-sensitive folding intermediates but relatively high final concentrations of folded protein (Rudolph 1990), thus facilitating detection. Additionally, two conditions using the detergent/cyclodextrin folding system were tested (Rozema and Gellman 1996; Hanson and Gellman 1998). Folding with this system comprises two steps. First, the denatured protein is diluted into a buffer containing the detergent CTAB. The protein is captured in a CTAB-protein complex, thus preventing aggregation. In the second step, refolding is initiated by adding β -cyclodextrin, which removes the detergent from the protein. Eight conditions for folding of His-tagged proteins on metal chelate resins were also included in the screen. The screen was automated using a pipetting robot.

Refolding of MDH

Malate dehydrogenase from pig heart (MDH) is a homodimeric enzyme of 36.5-kD subunits. It was successfully refolded using biological chaperones, with a yield of 60%–70% recovered active enzyme compared to less than 5% in the absence of chaperones (Weber et al. 1998).

MDH was refolded with the described automated refolding screen after denaturation in 7 M GdmHCl (conditions 1–22, Table 1). Conditions without glycerol resulted in no significant recovery of enzymatic activity ($\leq 2\%$ yield of active MDH). Most beneficial for the folding in the presence of glycerol was EDTA (conditions 11, 59% recovered activity). In addition, a low pH (condition 14, 37%) and the presence of the redox pair GSH/GSSG (condition 17, 33%) favored refolding of MDH.

For many proteins a biological assay may not be available as a folding monitor. We tried to find sensitive biophysical folding monitors that can be applied generally after folding screening. MDH is not usable for analysis with tryptophan fluorescence spectroscopy because it contains no such residues. In a new approach, an analytical HIC was tested as a folding monitor. Ammonium sulfate was added to all refolded samples before application onto a small column

Table 1. Composition of the automated folding screen

Condition	Additives for rapid dilution and CTAB/cyclodextrin screening for CAB, MDH, and p22 dynactin
1	—
2	0.8 M urea
3	1.6 M urea
4	0.03% PEG 1000
5	0.03% PEG 1000 + 0.8 M urea
6	0.03% PEG 1000 + 1.6 M urea
7	39% glycerol
8	39% glycerol + 0.8 M urea
9	EDTA ^(a)
10	EDTA + 0.03% PEG 1000 + 0.8 M urea ^(a)
11	EDTA + 39% glycerol ^(a)
12	20 mM citrate, pH 5.3 ^(b)
13	20 mM citrate, pH 5.3 + 0.03% PEG 1000 ^(b)
14	20 mM citrate, pH 5.3 + 39% glycerol ^(b)
15	5 mM/0.5 mM GSH/GSSG
16	5 mM/0.5 mM GSH/GSSG + 0.03% PEG 1000 + 0.8 M urea ^(c)
17	5 mM/0.5 mM GSH/GSSG + 39% glycerol ^(c)
18	20 mM citrate, pH 5.3 + 0.8 M urea ^(b)
19	0.75 M arginin
20	0.75 M arginin, 5 mM/0.5 mM GSH/GSSG ^(c)
21	0.57 mM CTAB/4.71 mM β -cyclodextrin
22	0.57 mM CTAB/4.71 mM β -cyclodextrin, 5 mM/0.5 mM GSH/GSSG ^(c)

Condition	Folding of His-tagged p22 dynactin on metal chelate resins
23	Folding on NiNTA-agarose, direct removal of urea
24	Folding on NiNTA-agarose, stepwise dilution of urea (8 M, 4 M, 2 M, 1 M)
25	Folding on Fractogel EMD Chelate, direct removal of urea
26	Folding on Fractogel EMD Chelate, stepwise dilution of urea (8 M, 4 M, 2 M, 1 M)
27	Folding on NiNTA-agarose, direct removal of urea + 0.03% PEG 1000
28	Folding on Fractogel EMD Chelate, direct removal of urea + 0.03% PEG 1000
29	Folding on NiNTA-agarose, direct removal of urea + 0.1 mM $\text{CaCl}_2/\text{MgCl}_2$
30	Folding on Fractogel EMD Chelate, direct removal of urea + 0.1 mM $\text{CaCl}_2/\text{MgCl}_2$

Conditions 1–22 contain 20 mM Tris, pH 7.7, 50 mM NaCl, 0.2 mM $\text{CaCl}_2/\text{MgCl}_2/\text{ZnCl}_2$ (when screening His-tagged p22 dynactin no ZnCl_2 was used), 1 mM DTT except ^(a) = no $\text{CaCl}_2/\text{MgCl}_2/\text{ZnCl}_2$, ^(b) = no Tris, ^(c) = no DTT. Folding buffer for condition 23–30 is 20 mM Tris, pH 7.7, 30 mM NaCl, 2 mM β -mercaptoethanol with or without additives, as indicated.

packed with a hydrophobic resin. Bound protein was eluted in a decreasing ammonium sulfate gradient. All conditions that resulted in no activity did not show an elution peak in HIC (e.g., conditions 1 and 4), whereas refolded MDH eluted in a close range from 49 to 53 mS from the HIC column (Fig. 1A). A linear correlation between recovered activity and peak height was observed, whereas protein yield (after removal of precipitates) was not correlated to activity (Fig. 1B).

Figure 1. Analysis of refolded MDH with HIC. (A) Recovered enzymatic activity and elution profiles of selected refolding conditions (see Table 1). (B) Correlation of recovered activity to peak heights (filled diamonds) observed during HIC and to protein yields (open squares; 100% yield corresponds to 45 $\mu\text{g/mL}$) for conditions 1–18; precipitates removed by centrifugation; conditions 19–22 excluded because arginine and CTAB are incompatible with the Bradford assay used to determine protein concentrations.

Refolding of CAB

CAB is a monomeric 28.5-kD enzyme with seven tryptophan residues and a zinc ion in its catalytic center. With this model enzyme we repeated the folding monitoring with analytical HIC that had been applied to MDH and additionally tested tryptophan fluorescence spectroscopy. Figure 2A shows the elution profiles during HIC and the yield of enzymatic activity (inset) of 4 of the 22 conditions tested for the refolding of GdmHCl-unfolded CAB. All 22 chromatograms of HIC could be divided into three groups: (1) no peak observed, (2) peaks at approximately 125 mS, and (3) peaks at approximately 111 mS. Only conditions 9, 10, and 11 belonged to group 3. These three conditions were the only ones that contained EDTA instead of ZnCl_2 (Table 1). Hence, HIC could discriminate CAB molecules with a zinc ion bound from molecules with no zinc ion.

From the fluorescence spectra of refolded CAB (Fig. 2B) we evaluated (1) the blue-shift of the fluorescence intensity

maximum compared to the denaturant-unfolded protein, and (2) the fluorescence intensity at 340 nm. The GdmHCl-unfolded CAB had a fluorescence intensity maximum of 355 nm. Spectra of refolded CAB that exhibited fluorescence had intensity maxima ranging from 355 nm down to 346 nm.

Figure 3A summarizes all data obtained from HIC, tryptophan fluorescence spectroscopy, and the recovery of enzymatic activity. Conditions 3 to 6 showed the highest fluorescence intensities of all tested conditions and, together with other samples (conditions 11 and 21), the strongest blue-shifts of the fluorescence intensity maxima (9 nm). The peak eluted from HIC at 125 mS for conditions 3, 4, 5, and 6 was the highest, the seventh highest, the fourth highest, and the third highest, respectively. These four conditions also showed the highest recovery of enzymatic activity (61%, 41%, 56%, and 67%, respectively). It can be concluded that CAB can be refolded most efficiently in the presence of PEG 1000 and/or urea. This beneficial effect was reported previously (Cleland et al. 1992b).

Figure 2. Analysis of refolded CAB with HIC and tryptophan fluorescence spectroscopy. (A) Elution profiles and recovered enzymatic activity (inset) of selected refolding conditions (see Table 1). (B) Fluorescence emission spectra (upon excitation at 280 nm) of the refolding conditions shown in (A) and of denatured CAB in 6 M GdmHCl (120 $\mu\text{g/mL}$), indicated with “D”.

Figure 3. Correlation of recovered activities of refolded CAB to peak heights observed during HIC, fluorescence intensities, blue-shifts of maximum fluorescence intensities, and protein yields. (A) Schematic depiction of the different parameters (recovered enzymatic activity, peak heights observed during HIC at 125 mS, fluorescence intensities, blue-shifts of maximum fluorescence intensity, and protein yields [after removal of precipitates with filtration]) measured for the tested conditions; *Detected peaks during HIC eluted at 111 mS (see text and Fig. 2). (B) Correlation of recovered activities to the folding signals obtained with peak heights of HIC at 125 mS (filled squares), fluorescence intensities (gray diamonds), blue-shifts of maximum fluorescence intensities (open triangles), and to protein yields (crosses; 100% yield corresponds to 120 $\mu\text{g/mL}$); conditions 9–11 are excluded due to a different behavior in HIC (eluted peak at 111 mS). Protein yields calculated from A_{280} , with correction for stray light using A_{340} ; note that despite this correction, for samples denoted with “a” the strong stray light gave rise to yields higher than 100%. Fluorescence intensities were measured at 340 nm upon excitation at 280 nm. All values given as relative units with respect to the conditions that resulted in the highest value obtained with the respective folding monitor.

In Figure 3B the recovery of enzyme activity is correlated to four parameters: (1) peak height at 125 mS during HIC, (2) fluorescence intensity, (3) blue-shift of the fluorescence intensity maximum, and (4) protein yield. Conditions 9–11 of the folding screen were excluded from the graph due to their behavior in HIC, as described above and shown in Figure 2A. Fluorescence intensity showed a linear correlation to recovered activity, with the exception of condition 17 (a false positive), which had high intensity but no enzymatic activity. The values of the HIC peak heights could also be correlated linearly to activity. However, three false positives occurred (conditions 7, 8, and 17, compare with Fig. 3A). Additionally, conditions 16 and 21 could be considered as false negatives, because some enzymatic activity was detected (both 9%), but HIC showed no elution peak. For all samples with a recovered enzymatic activity higher than 15%, significant blue-shifts of fluorescence intensity maxima could be observed (Fig. 3B). Among the samples with less than 15% recovered activity shown in Figure 3B (seven data points), five samples showed small or no blue-shifts. The two remaining samples exhibited strong blue-shifts; one of them showed no recovered activity (condition 17), and the second (condition 21, Fig. 3A) had 9% recovered activity. Protein yield was the least reliable of the four parameters for analyzing the refolding screen of CAB (Fig. 3B).

Figure 3A demonstrates that the three monitors were useful in conjunction, to rule out false positives or false negatives that were obtained with one monitor. Conditions 7 and 8 exhibited no fluorescence signal and correspondingly led to almost no activity yield (2% and 1%, respectively) even though peaks at 125 mS were observed during HIC. On the other hand, no recovered activities were observed for con-

ditions 9 and 11, even though fluorescence was detected as well as a blue-shift of the fluorescence intensity maximum. However, these samples could be ruled out due to their deviating behavior in HIC. Condition 17 was the only condition showing no enzymatic activity, but positive signals with all three monitors. Considering all results, no false negatives occurred, because all conditions that led to enzymatic activity were either clearly detected by analytical HIC or showed a blue-shift in fluorescence spectroscopy.

Interestingly, the beneficial effect of the CTAB/cyclodextrin system on the refolding of CAB reported before (Rozema and Gellman 1996) was not observed in our screen. This could be due to the higher protein concentration used in this study during refolding (120 $\mu\text{g/mL}$, compared to 30 $\mu\text{g/mL}$).

Screening for folding conditions for p22 dynactin

After evaluation of the automated folding screen and the biophysical detection system for folded protein with two model enzymes, the screening system was applied to the folding of the human protein p22 dynactin that is deposited in inclusion bodies upon expression in *E. coli*. Lowering the temperature from 37°C to 22°C during protein expression did not result in soluble expression (data not shown).

p22 dynactin was expressed with an N-terminal His-tag, followed by a TEV protease cleavage site. Inclusion body protein was solubilized with GdmHCl, purified by metal chelate chromatography under denaturing condition and submitted to the folding screen and to the biophysical analyses. The His-tag was not only used for purifying the protein prior to the folding screen, but also to test folding of p22

dynactin immobilized on metal chelate resins (Table 1, conditions 23–30). During this procedure, an interference of the folding protein molecule with the chromatographic resin can be counterproductive for refolding. This interference was shown to be dependent on the salt concentration when folding poly-Arg-tagged α -glucosidase on heparin sepharose (Stempfer et al. 1996). In this study, a NaCl concentration in the range of 30 mM gave the best results for folding. Therefore, we chose this salt concentration in our attempts to fold His-tagged p22 dynactin on NiNTA-agarose or Fractogel EMD Chelate loaded with Ni^{2+} .

Figure 4, A and B, shows the results of three conditions that resulted in a peak during HIC, plus condition 26 with no peak. All peaks during HIC eluted at approx. 138 mS. The highest peaks were observed with the CTAB/cyclodextrin conditions 21 and 22, as well as with condition 19 and condition 7 (Fig. 5A). Conditions 7, 19, and 21 also showed a strong blue-shift of the fluorescence maximum compared to the denatured p22 dynactin. The sample obtained from condition 26 had no peak in HIC but exhibited strong fluorescence and a significant blue-shift, as shown in Figure 4B. It has to be noted that more denatured protein was used for the conditions 23–30 (folding of p22 immobilized on metal chelate resins) than for 1–22 (folding by rapid dilution). Therefore, fluorescence intensities between those conditions could not be compared directly. Intensities of rapid dilution samples were relatively low due to the fact that dynactin only contains two tryptophans.

Figure 5, A and B, shows that conditions 2, 7, 19, 21, and 22 showed “positive” signals for all measured parameters, with condition 2 showing the lowest signals. For condition 22, a comparably small blue-shift of the fluorescence maximum was observed.

From these results, it can be assumed that p22 dynactin can be folded using glycerol, arginine, or the CTAB/cyclodextrin system. The presence of 5 mM/0.5 mM GSH/GSSG seems to be counterproductive. None of the samples of re-

folding on the metal chelate supports gave a peak in HIC, even though their fluorescence intensities were high and a significant blue-shift could be observed—especially for the samples that had been refolded on Fractogel EMD Chelate. Hence, as with MDH (see above), HIC seems to be a good “knock-out” criterion for analyzing the folding screen for p22 dynactin.

Folding, purification, and CD-analysis of p22 dynactin

We purified the p22 dynactin on a large scale using the condition 21. Inclusion bodies resulting from a 4-L *E. coli* culture were solubilized and His-tagged p22 dynactin was purified using metal chelate chromatography under denaturing conditions. After addition of cyclodextrin to the p22 dynactin/CTAB mixture to induce folding, TEV protease was added to remove the His-tag, and the sample was incubated overnight at 18°C. Folded p22 was then purified as described in Materials and Methods, yielding 25 mg protein. The size detected during size-exclusion chromatography corresponded to monomeric protein. Figure 6A shows a SDS-PAGE of the urea-denatured and the purified p22 dynactin. The major impurity after folding and purification was a band at 17 kD, which was a degradation product of p22 dynactin that was formed during the incubation time of the folding reaction. It was not a product of TEV-protease cleavage but seemed to result from a protease that was folded in small amounts together with p22 dynactin. This protease seemed to be separated from p22 dynactin during purification. The truncated p22 dynactin could not be entirely removed during the purification procedure.

The preparation was analyzed using CD spectroscopy. A near-UV spectrum of p22 dynactin (Fig. 6B) exhibited a pronounced fine structure, showing that the aromatic residues were immobilized in an asymmetric environment and that the protein attained a well-defined, stable fold. The far-

Figure 4. Analysis of folded p22 dynactin with HIC and fluorescence spectroscopy. (A) Elution profiles from HIC column of conditions 2, 7, 19, 21, and 22 determined from absorbance at 280 nm. (B) Fluorescence emission spectra (upon excitation at 280 nm) of the refolding conditions shown in (A) and of denatured dynactin (8 M urea, 100 $\mu\text{g/mL}$), indicated with “D”.

Figure 5. Peak heights during HIC, fluorescence intensities, and blue-shifts of maximum fluorescence intensities of folded p22 dynactin. (A) Rapid dilution conditions 1–22. (B) Folding on NiNTA and Fractogel EMD Chelate (conditions 23–30). Peak heights from HIC only included in (A) because conditions 23–30 showed no peak in HIC. Fluorescence intensities were measured at 340 nm upon excitation at 280 nm. All values given as relative units with respect to the conditions that resulted in the highest value obtained with the respective folding monitor. Rapid dilution conditions and the conditions for folding on metal chelate resins are shown separately because different amounts of denatured protein were used for those two groups of conditions.

UV spectrum (Fig. 6C) indicated high α -helix content, in accordance with secondary structure prediction for p22 dynactin (63% α -helix, 9% β -sheet, calculated with PSIPRED). In accordance with the result from CD spectroscopy, analysis with FTIR spectroscopy suggested an α -helical fold (data not shown). p22 dynactin could be concentrated to 25 mg/mL without any occurrence of aggregation, as was proven by dynamic light scattering (a hydrodynamic diameter of 4 nm was observed).

Discussion

Automated folding screen

The folding of proteins that are expressed insolubly in *E. coli* cells is a major obstacle in proteomics projects as well as in general biochemical research and in industrial processes that involve production of proteins as pharmaceuti-

cals. Screening for suitable conditions to fold proteins can be very labor-intensive. To facilitate this process, we implemented an automated folding screen and proved its efficiency by refolding MDH and CAB. The procedure is flexible, and the screen can easily be modified by reprogramming the robot or by exchanging stock solutions, for example, for altering the pH values. The screen facilitates folding of proteins by rapid dilution as well as folding of proteins that are immobilized on affinity resins. For the complete screen of 30 conditions, only 5 mg pure, denaturant-solubilized protein is needed. To our knowledge, this is the first published folding screen using a robot system.

During in vitro protein folding, yields are affected by multiple factors. A screen where all factors are checked in all possible combinations will result in a very high assay number. However, in a primary screen some factor combinations may be excluded. This exclusion can be done on the basis of known properties of particular factors, with respect

Figure 6. Analysis of folded, purified p22 dynactin with SDS-PAGE and CD spectroscopy. (A) Lane A, denatured protein after purification with Ni-POROS20 column; lane B, natively folded, purified protein. Due to removal of the His-tag with TEV-protease the native protein is 3 kD smaller than the denatured protein. M = molecular weight marker. (B) and (C) near- and far-UV-CD spectra, respectively. Sample concentration: 1.03 mg/mL.

to their effects on proteins. For example, disulfide bond formation is favored at basic pH (Misawa and Kumagai 1999). Hence, the combination of an acidic pH with a redox pair are unlikely to be beneficial in conjunction. In contrast, other additives proved to be beneficial in conjunction, like PEG with urea or GdmHCl (Cleland et al. 1992a, 1992b). Such assumptions and observations have been considered when composing the folding screen presented in this work (Table 1).

We chose a screening procedure in which only one parameter at a time is changed. Most of the other available screens consist of changes of many parameters at once. This approach is commonly known as fractional factorial screen (Armstrong et al. 1999). For fractional factorial screening, a functional assay or a reliable method for the quantification of correctly folded protein (e.g., the peak heights resulting from gel filtration experiments) is necessary for the calculation of response values for the screened factors (additives, pH, or protein concentration). The response values are used to determine whether a particular factor is beneficial for the folding of a certain protein. However, a functional assay or one stringent quantification method may often not be available. After a folding screen, protein concentrations are often too low for gel filtration experiments. In this work, we demonstrated that a reliable evaluation of a folding screen can sometimes only be accomplished by monitoring more than one (biophysical) parameter. Such a multidata analysis procedure seems to be difficult to be applied to the evaluation strategy used for fractional factorial screening. Another disadvantage of a fractional factorial screen is that interferences between the different additives may distort the factor calculation. Armstrong et al. (1999) refolded CAB with a fractional factorial screen and found a negative effect of PEG, even though the beneficial effect of PEG was shown before (Cleland et al. 1992b) and was confirmed in this work. The authors attributed this observation to multi-factor interactions (Armstrong et al. 1999).

Combination of biophysical methods for folding detection

We addressed the problem of reliable and rapid detection of small amounts of natively folded protein from a folding screen when no biological assay is available for the analyzed protein. This problem is particularly important for proteomics projects that deal with large numbers of diverse proteins, but may also be inherent in various other fields where a functional test to detect native protein is not available or very time- and/or product-consuming. This work shows for two proteins analyzed, CAB and p22 dynactin, that tryptophan fluorescence spectroscopy can be helpful to evaluate the outcome of a folding screen. From the fluorescence spectra we examined (1) the blue-shift of the fluorescence intensity maximum compared to the denaturant-un-

folded protein, and (2) the fluorescence intensity. As explained above (see introduction), fluorescence intensities should only be used in conjunction with other monitors. False negatives did not occur, as all folding conditions that contained folded protein, that is, that showed CAB activity or, in the case of p22 dynactin, gave a peak in analytical HIC, were indeed detected. It was shown during large-scale preparation that the p22 dynactin peak eluted from HIC represented folded protein, presumably in the native conformation, according to detailed biophysical characterization.

However, when detecting folded p22 dynactin with fluorescence spectroscopy, some samples with high intensities as well as significant blue-shifts were observed, even though there was no peak in HIC. Misfolded microaggregates formed after a folding experiment can be structurally very compact, thereby mimicking the fluorescence properties of native protein and giving rise to false positives. Another limitation of tryptophan fluorescence spectroscopy is that some proteins cannot be analyzed because they do not contain tryptophan residues or the tryptophan fluorescence has a low quantum yield. Therefore, an alternative method to fluorescence spectroscopy should be employed.

In a new approach, we showed that HIC can be a powerful tool to judge the outcome of a folding screen. Adding an appropriate amount of ammonium sulfate and performing HIC can be used to separate misfolded from natively folded protein as shown for MDH, CAB, and p22 dynactin. In almost all cases, natively folded proteins showed a distinct peak in HIC due to their conformational uniformity. Misfolded or aggregated protein might (1) precipitate upon the addition of ammonium sulfate, (2) bind irreversibly to the column material, or (3) be eluted erratically from the column. The yield of natively folded protein can be estimated quite reliably by simply comparing peak heights. Less than 20 μ g of natively folded protein could be detected for the analyzed proteins. Interestingly, HIC could detect the binding of the metal cofactor zinc to CAB. Although one would expect only a small effect on the structure of CAB caused by binding of zinc, Zn-CAB elutes earlier from the HIC column and is presumably a less hydrophobic molecule.

The type of HIC resin needs some consideration. For small globular proteins, up to 50 kD, a resin with relatively high hydrophobicity should be used. We noticed a rough correlation between protein size and the binding strength, with bigger proteins binding stronger than smaller proteins (data not shown). Hence, in most cases, smaller proteins need a higher concentration of ammonium sulfate to bind to the column. However, if the concentration is too high, the target protein might precipitate. To find an optimum concentration, we suggest that two concentrations are tested: first, a low ammonium sulfate concentration (e.g., 1.5 M). If no peaks are detected with aliquots tested in these runs, more ammonium sulfate can be added and HIC is repeated.

Ideally, a detection method for folded protein upon folding screening should lead to neither false negatives nor false positives. False negatives are highly detrimental because they lead to a complete loss of a positive sample and should therefore be avoided by all means. False positives can always be excluded later by a more detailed investigation. With the folding screen analysis of CAB and p22 dynactin, we demonstrated how two biophysical folding monitors, analytical HIC and tryptophan fluorescence spectroscopy, can be used in conjunction to rule out false positives or negatives obtained with one of the respective monitors. In the case of CAB, one condition (number 17) resulted in no enzymatic activity, but a peak during HIC as well as a blue-shift of the fluorescence intensity maximum was observed. As small structural changes may result in complete loss of activity, we assume that the refolded product had a slightly different structure than native CAB.

By automation and miniaturization we could facilitate protein folding screening. We implemented a sensitive biophysical detection system for native protein, thus making folding screening also adaptable to proteins for which no functional assays are available. For all three proteins investigated we managed to find effective conditions for (re-)folding. The folding of p22 dynactin was successfully scaled up to yield an amount of protein that is enough for detailed analysis and crystallization trials. The presented approach might help to overcome well-known obstacles encountered during protein folding and facilitate the availability of inclusion body proteins for proteomics.

Materials and methods

Secondary structure prediction of p22 dynactin was performed with PSIPRED (Department of Computer Science, University College London).

Bovine erythrocyte carbonic anhydrase II (CAB) was purchased from Sigma, pig heart malate dehydrogenase (MDH) from Merck. Protein concentrations were determined from the absorbance at 280 nm using the extinction coefficient calculated from the amino acid sequence (Mach et al. 1992). Absorbance was corrected for stray light according to the light-scattering theory (Tyndall effect, $I_{(s)} \sim \lambda^{-4}$), with the assumption that no absorption due to protein chromophores occur above 320 nm (Levine and Federici 1982). For protein concentration determination of conditions 1–18 of refolded MDH, 250- μ L aliquots were centrifuged at $25,000 \times g$ for 30 min at 8°C, and supernatants were analyzed with the Bio-Rad Bradford Assay with native MDH as the standard. Arginine and CTAB present in conditions 19–22 are not compatible with this assay.

CAB and MDH were denatured for 5 h at room temperature. Conditions used were: (1) 6 mg/mL CAB in 20 mM Tris-HCl, pH 7.7, containing 6 M GdmHCl and 2 mM EDTA; and (2) 2.24 mg/mL MDH in 20 mM Tris-HCl, pH 7.7, containing 7 M GdmHCl and 10 mM DTT.

Expression of recombinant p22 dynactin in *E. coli*

p22 dynactin (GenBank accession AAC61280) was expressed at 37°C in *E. coli* SCS1 cells using the pQTEV expression vector

(GenBank accession AY243506). SCS1 cells contained the Rosetta helper plasmid (Novagen). Proteins expressed from the pQTEV vector carried an N-terminal heptahistidine-tag (His-tag) followed by a TEV-protease cleavage site. A 100-mL overnight culture was prepared in 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0), supplemented with 2% glucose, 100 μ g/mL ampicillin, and 30 μ g/mL chloramphenicol. This culture was transferred into 1.9 L of SB medium (12 g/L Bacto-tryptone, 24 g/L yeast extract, 0.4% [v/v] glycerol, 17 mM KH_2PO_4 , 72 mM K_2HPO_4), supplemented with 20 μ g/mL thiamine, 100 μ g/mL ampicillin, and 30 μ g/mL chloramphenicol. At an OD_{600} of 1.5, protein expression was induced for 4 h by adding IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation ($4000 \times g$ for 20 min at 4°C) and washed with 20 mM Tris-HCl, pH 7.7, 150 mM NaCl. They were frozen in liquid N_2 and stored at -80°C until use.

Inclusion body preparation and denaturing His-tag purification of p22 dynactin

Cells were suspended in ice-cold 20 mM Tris-HCl, pH 7.7, 300 mM NaCl, 2 mM DTT, 1 mM EDTA, before disruption by sonification. The crude extract was centrifuged at $25,000 \times g$ for 45 min, 10°C , and the pellet was washed twice in 20 mM Tris-HCl, pH 7.7, 300 mM NaCl. The resulting pellet was dissolved in 20 mM Tris-HCl, pH 7.7, 6.6 M GdmHCl, 30 mM NaCl, 5 mM imidazole, 2 mM β -mercaptoethanol overnight under stirring at room temperature. The GdmHCl-insoluble material was removed by centrifugation ($25,000 \times g$, 60 min) and the supernatant was applied to a 7.8-mL Ni-POROS20-column (Applied Biosystems), previously equilibrated in urea buffer (20 mM Tris-HCl, pH 7.7, 8 M urea, 30 mM NaCl, 5 mM imidazole, 2 mM β -mercaptoethanol). After washing with 15 column volumes of urea buffer, bound protein was eluted using urea buffer enriched with 300 mM imidazole. The protein concentration was adjusted to 5 mg/mL by dilution into urea buffer. EDTA and DTT was added to achieve final concentrations of 1 mM and 15 mM, respectively, followed by a 60-min incubation at room temperature to reduce cysteines. The obtained protein solution was used for rapid dilution screen conditions (Table 1). For screening conditions where p22 dynactin is immobilized on NiNTA agarose or Fractogel EMD Chelate during folding, EDTA, DTT, and imidazole had to be removed by a buffer exchange into urea buffer using a HiTrap desalting column (Amersham Bioscience). p22 dynactin (1.2 mg) was incubated for 20 min while shaking with 350 μ L NiNTA agarose or 350 μ L Fractogel EMD Chelate, loaded with Ni^{2+} , both equilibrated in urea buffer. After binding, the suspension with the affinity resins was aliquoted in eight portions, four for each resin. After the resins had settled at the bottom of the tubes, the supernatant with unbound protein was removed leaving 100 μ L in every tube.

Automated folding screening

The pipetting robot used (Speedy, Zinsser Analytic AG) has four steel pipetting needles and is equipped with a temperature-controlled plate that keeps all solutions at 16°C – 18°C . Protein folding was carried out in a rack containing 2-mL tubes for conditions 1–22 (see Table 1) and 1.5-mL tubes for folding of p22 dynactin that was immobilized on metal chelate resins (conditions 23–30, Table 1). All buffer components for rapid dilution screening were pipetted together from stock solutions. For protein addition steps, the rack was transferred onto a stirrer to ensure effective mixing.

Screening conditions 1–22 had a final volume of 1 mL. For conditions 1–20, 20- μ L aliquots of denatured protein were added in four steps, divided by 150-min incubation time. In the case of conditions 21 and 22 (CTAB/cyclodextrin system), protein was added in one step to 680 μ L CTAB-containing buffer and incubated for 150 min, followed by addition of 300 μ L β -cyclodextrin and a further 10-h incubation; 350 μ L aliquots were submitted to dialysis and subsequent fluorescence spectroscopy and 400 μ L were applied to HIC (see below).

For conditions 23–30, denatured protein bound to affinity beads (prepared as described above) was washed with 1.3 mL urea buffer (see above). All conditions, except for 24 and 26, were washed three times in 1.3 mL folding buffers and incubated for 10 h to induce folding. For conditions 24 and 26, the urea concentration was stepwise decreased by dilution with folding buffer (see Table 1, 15-min intervals) followed by two washes and a 9-h incubation. In summary, all samples were washed five times in total, including a final transfer into the same buffer (20 mM Tris-HCl, pH 7.7, 30 mM NaCl, 2 mM β -mercaptoethanol). Finally, protein was eluted with 400 μ L of 20 mM Tris-HCl, pH 7.7 150 mM NaCl, 2 mM β -mercaptoethanol, 500 mM imidazole. The obtained samples were analyzed by fluorescence spectroscopy before application to the analytical HIC column (see below).

Tryptophan fluorescence spectroscopy

Three hundred fifty microliters of samples of conditions 1–22 of the folding screen were dialyzed for 2 days against 20 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, using a membrane with a 4000–6000-Dalton cutoff (Zellu Trans, Roth). Samples obtained from conditions 23–30 were not dialyzed. Three hundred twenty microliters of all samples were filtrated through a 96-well 0.22- μ m PVDF membrane (Millipore) and collected in a black 96-well plate (Corning) that has a UV-transparent bottom. A_{280} and A_{340} were measured before fluorescence emission spectra were taken between 285 nm and 420 nm upon excitation at 280 nm (Cary Eclipse, Varian Instruments). The reference spectrum was subtracted from the data.

Hydrophobic interaction chromatography (HIC)

To 400 μ L aliquots from the folding screen (see above) 3.8 M ammonium sulfate was added under vigorous stirring to achieve a final ammonium sulfate concentration of 2.0 M. Samples were centrifuged (30 min, 25,000 $\times g$, 8°C) and applied to a POROS HP2 column (0.83 mL bed volume, Applied Biosystems), equilibrated in 20 mM Tris-HCl, pH 7.7, 2.0 M ammonium sulfate. Chromatography was performed at 8°C (Vision workstation, Applied Biosystems). The column was washed with equilibration buffer and a gradient to 0 M ammonium sulfate was performed over 18, 14, or 10 column volumes for CAB, MDH, and p22 dynactin, respectively. After each run, the column was washed with 5 M GdmHCl to regenerate the column. The flow rate was 3.5 mL/min; each run took approximately 15 min. The relatively low absorption coefficient at 280 nm ($\epsilon = 10,264 \text{ (M cm)}^{-1}$) of the 36.6-kD monomeric MDH prompted us to use the absorbance at 220 nm to detect elution peaks from HIC for this protein. All shown chromatograms were baseline corrected.

Determination of CAB and MDH activity

CAB activities were measured using the pNPAc esterase assay (Rozema and Gellman 1996). Fifty microliters of dialyzed samples

from the refolding screen were pipetted into 400 μ L 20 mM Tris-HCl, pH 7.7. After addition of 45 μ L of 52 mM pNPAc in dry acetonitril and mixing, the formation of the hydrolysis product, *p*-nitrophenolate, was monitored by measuring the linear increase in absorbance at 400 nm from 30 to 60 sec (background hydrolysis was subtracted). MDH activities were assayed as described before (Hutchinson et al. 1994). The measured activities for both CAB and MDH were related to the activity of a control sample with native enzymes. Absorbance changes were measured using a spectrophotometer (Cary 50 Scan, Varian instruments).

Large-scale purification of p22 dynactin

Inclusion bodies of p22 dynactin from 4-L cultures were prepared and purified as described above. Fifteen milliliters of pure denatured protein (5 mg/mL) in 20 mM Tris-HCl, pH 7.5, 8 M urea, 30 mM NaCl, 15 mM DTT, 1 mM EDTA, 300 mM imidazole was diluted into 510 mL of 20 mM Tris-HCl, 50 mM NaCl, 0.2 mM $\text{CaCl}_2/\text{MgCl}_2$, 0.57 mM CTAB, 2 mM β -mercaptoethanol under vigorous stirring. After 2-h incubation, 225 mL of 15.7 mM β -cyclodextrin was added under vigorous stirring to induce folding at 18°C. After 1 h, 7.5 mg TEV-protease was added, followed by an overnight incubation to cut off the His-tag and to ensure that the folding reaction is completed. After centrifugation to remove precipitates, the folded protein was applied to anion exchange chromatography (POROS HQ, Applied Biosystems) where p22 dynactin did not bind. Subsequently, the p22 dynactin/TEV protease mixture was applied to NiNTA-superose (Qiagen) to remove the TEV-protease. To the flowthrough containing p22 dynactin, 3.8 M ammonium sulfate was added under vigorous stirring to achieve a final ammonium sulfate concentration of 2.2 M. The resulting 2.2 L were applied onto a POROS HP2 column (7.8-mL bed volume) and purified as described above. The protein eluate from the ammonium sulfate gradient was applied to a Superose 12 16/50 column (Pharmacia), equilibrated in 20 mM Tris-HCl, pH 7.7, 50 mM NaCl. Eluted protein fractions that correspond to monomeric p22 dynactin (determined using standard molecular weight markers) were collected. A yield of 25 mg of p22 dynactin was obtained in 25 mL, representing 33% of the urea-denatured protein subjected to the purification procedure. An aliquot of the gel filtration eluate was directly analyzed with CD spectroscopy. The protein was concentrated to a final concentration of 24.7 mg/mL by ultrafiltration (Biomax centrifuge tubes, Millipore). It was examined for occurrence of aggregation, by DLS using the Laser SpectroScatter201 (RiNA GmbH, Germany). The purity of the sample was assessed by SDS-PAGE (Laemmli 1970), using Coomassie staining.

UV CD spectroscopy

A Jasco 715 CD spectropolarimeter, calibrated with camphor sulfonic acid, was used for the CD experiment. The concentration of p22 dynactin in 20 mM Tris/Cl, 50 mM NaCl, pH 7.7 was 1.03 mg/mL. Data were averaged at 10°C over 25 scans accumulated at 0.5-nm intervals with 50-nm/min scan speed and 1-sec response time. In the far UV, overlapping spectra were measured in 1 mm (250–207 nm) and 0.1 ± 0.005 mm (250–186 nm) path-length cells (Hellma, Müllheim, Germany). Spectra were baseline corrected, and the overlapping part of the spectra was used to correct for variations in the path length of the 0.1-mm cell. The spectrum in the near-UV region (350–250 nm) was measured in a 4-mm path-length cell calibrated by absorption spectroscopy.

Acknowledgments

We are thankful to Dr. Volker Sievert for production of the expression clone for p22 dynactin and to Dr. Monika Walter for help with the CD spectra. This work was funded by the German Federal Ministry of Education and Research (BMBF) through the "Leitprojektverbund Proteinstrukturfabrik."

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Armstrong, N., de Lencastre, A., and Gouaux, E. 1999. A new protein folding screen: Application to the ligand binding domains of a glutamate and kainate receptor and to lysozyme and carbonic anhydrase. *Protein Sci.* **8**: 1475–1483.
- Chen, G.Q. and Gouaux, E. 1997. Overexpression of a glutamate receptor (GluR2) ligand binding domain in *Escherichia coli*: Application of a novel protein folding screen. *Proc. Natl. Acad. Sci.* **94**: 13431–13436.
- Clark, E.D. 2001. Protein refolding for industrial processes. *Curr. Opin. Biotechnol.* **12**: 202–207.
- Cleland, J.L. and Wang, J.L. 1990. Refolding and aggregation of bovine carbonic anhydrase B: Quasi-elastic light scattering analysis. *Biochemistry* **29**: 11072–11078.
- Cleland, J.L., Builder, S.E., Swartz, J.R., Winkler, M., Chang, J.Y., and Wang, D.I. 1992a. Polyethylene glycol enhanced protein refolding. *Biotechnology (NY)* **10**: 1013–1019.
- Cleland, J.L., Hedgepeth, C., and Wang, D.I. 1992b. Polyethylene glycol enhanced refolding of bovine carbonic anhydrase B. Reaction stoichiometry and refolding model. *J. Biol. Chem.* **267**: 13327–13334.
- Das, B.K. and Liang, J.J. 1998. Thermodynamic and kinetic characterization of calf lens gammaF-crystallin. *Int. J. Biol. Macromol.* **23**: 191–197.
- Fink, A.L. 1998. Protein aggregation: Folding aggregates, inclusion bodies and amyloid. *Fold. Des.* **3**: R9–23.
- Gill, S.R., Schroer, T.A., Szilak, I., Steuer, E.R., Sheetz, M.P., and Cleveland, D.W. 1991. Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. *J. Cell Biol.* **115**: 1639–1650.
- Guise, A.D., West, S.M., and Chaudhuri, J.B. 1996. Protein folding in vivo and renaturation of recombinant proteins from inclusion bodies. *Mol. Biotechnol.* **6**: 53–64.
- Hanson, P.E. and Gellman, S.H. 1998. Mechanistic comparison of artificial-chaperone-assisted and unassisted refolding of urea-denatured carbonic anhydrase B. *Fold. Des.* **3**: 457–468.
- Heiring, C. and Muller, Y.A. 2001. Folding screening assayed by proteolysis: Application to various cysteine deletion mutants of vascular endothelial growth factor. *Protein Eng.* **14**: 183–188.
- Hutchinson, J.P., el-Thaher, T.S., and Miller, A.D. 1994. Refolding and recognition of mitochondrial malate dehydrogenase by *Escherichia coli* chaperonins cpn 60 (groEL) and cpn10 (groES). *Biochem. J.* **302**: 405–410.
- Karki, S., LaMonte, B., and Holzbaur, E.L. 1998. Characterization of the p22 subunit of dynactin reveals the localization of cytoplasmic dynein and dynactin to the midbody of dividing cells. *J. Cell Biol.* **142**: 1023–1034.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Levine, R.L. and Federici, M.M. 1982. Quantitation of aromatic residues in proteins: Model compounds for second-derivative spectroscopy. *Biochemistry* **21**: 2600–2606.
- Lilie, H., Schwarz, E., and Rudolph, R. 1998. Advances in refolding of proteins produced in *E. coli*. *Curr. Opin. Biotechnol.* **9**: 497–501.
- Mach, H., Middaugh, C.R., and Lewis, R.V. 1992. Detection of proteins and phenol in DNA samples with second-derivative absorption spectroscopy. *Anal. Biochem.* **200**: 20–26.
- Middelberg, A.P. 2002. Preparative protein refolding. *Trends Biotechnol.* **20**: 437–443.
- Misawa, S. and Kumagai, I. 1999. Refolding of therapeutic proteins produced in *Escherichia coli* as inclusion bodies. *Biopolymers* **51**: 297–307.
- Rehm, B.H., Qi, Q., Beermann, B.B., Hinz, H.J., and Steinbuchel, A. 2001. Matrix-assisted in vitro refolding of *Pseudomonas aeruginosa* class II polyhydroxyalkanoate synthase from inclusion bodies produced in recombinant *Escherichia coli*. *Biochem. J.* **358**: 263–268.
- Royer, C.A. 1995. Fluorescence spectroscopy. In *Methods in molecular biology* (ed. B.A. Shirley), pp. 65–90. Humana Press, Totowa, NJ.
- Rozema, D. and Gellman, S.H. 1996. Artificial chaperone-assisted refolding of carbonic anhydrase B. *J. Biol. Chem.* **271**: 3478–3487.
- Rudolph, R. 1990. *Renaturation of recombinant, disulfide-bonded proteins from inclusion bodies*. Walter de Gruyter, New York.
- Rudolph, R. and Lilie, H. 1996. In vitro folding of inclusion body proteins. *FASEB J.* **10**: 49–56.
- Stempfer, G., Holl-Neugebauer, B., and Rudolph, R. 1996. Improved refolding of an immobilized fusion protein. *Nat. Biotechnol.* **14**: 329–334.
- Tobbell, D.A., Middleton, B.J., Raines, S., Needham, M.R., Taylor, I.W., Beveridge, J.Y., and Abbott, W.M. 2002. Identification of in vitro folding conditions for procathepsin S and cathepsin S using fractional factorial screens. *Protein Expr. Purif.* **24**: 242–254.
- Weber, F., Keppel, F., Georgopoulos, C., Hayerhartl, M.K., and Hartl, F.U. 1998. The oligomeric structure of Groel/Groes is required for biologically significant chaperonin function in protein folding. *Nat. Struct. Biol.* **5**: 977–985.
- Zahn, R., von Schroetter, C., and Wuthrich, K. 1997. Human prion proteins expressed in *Escherichia coli* and purified by high-affinity column refolding. *FEBS Lett.* **417**: 400–404.