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The influence of mixing on lysozyme renaturation during refolding in an oscillatory flow and a stirred-tank reactor

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Abstract

Protein refolding is a key unit operation in many processes that produce recombinant biopharmaceuticals using *Escherichia coli*. Yield in this step generally controls overall process yield, and at industrially relevant protein concentrations is limited by aggregation. While most refolding operations are optimised with respect to chemical environment, the physical processes affecting yield have been neglected. In this study, we demonstrate that refolding yield for the model protein lysozyme is dependent on mixing intensity during dilution refolding. This is shown for two different reactor configurations: a standard stirred-tank reactor and a novel oscillatory flow reactor. We further show that the effect of mixing is dependent on the type of chaotrope employed for denaturation. Yield falls significantly when mixing intensity is decreased following urea denaturation, while the effect of mixing is not apparent when guanidine hydrochloride is employed as the denaturant. In batch tests we further confirm that, for urea, the "path" of dilution affects yield, and hence the observed sensitivity to mixing is not unexpected. We conclude that mixing is a critical parameter that must be optimised in industrial reactors, along with the usual chemical and protein-specific parameters.

Keywords: Mixing; Downstream processing; Protein refolding; Oscillatory flow; Renaturation

1. Introduction

Numerous recombinant proteins are expressed within *Escherichia coli* as a protein inclusion body. Such a process route offers advantages including high expression yield (Lee, 1996), protection from intracellular proteolysis, and simplified initial purification through centrifugal recovery of the recombinant inclusion bodies (Middelberg, 1996). However, these advantages are rapidly lost if the denatured protein contained in the inclusion bodies cannot be efficiently refolded to give biologically active protein.

Protein aggregation represents a major pathway for loss of product during refolding. Aggregation is typically suppressed by refolding at low protein concentrations in a batch system (Zettlmeissl, Rudolph, & Jaenicke, 1979; Goldberg, Rudolph, & Jaenicke, 1991) or by utilising a fed-batch or continuous reactor design, as is appropriate for a kinetic scheme characterised simplistically by competing first-order renaturation and higher-order aggregation (Middelberg, 1996; Kotlarski, O'Neill, Francis, & Middelberg, 1997). Regardless of the precise reactor strategy, refolding volumes are often large as protein concentrations are typically below 1 mg ml^{-1}. At typical process scale, refolding tanks are characterised by imperfect mixing and consequently protein and denaturant concentration gradients. As aggregation intermediates can accumulate with a time constant of seconds following dilution (Goldberg et al., 1991; Speed, Wang, & King, 1995), the protein and chemical gradients within the reactor might be expected to affect overall renaturation yield. However, while numerous studies have been directed toward optimizing the chemical environment for refolding a wide range of proteins, investigations into the impact of physical parameters such as mixing intensity are rare. There is nevertheless evidence that mixing intensity affects refolding yield, as demonstrated for lysozyme (Goldberg et al., 1991) and insulin-like growth factor (Kotlarski, 1998). Despite these reports, a physical understanding of the impact of mixing on refolding yield is presently lacking.

In this work we therefore investigate, and seek to explain, the impact of mixing intensity on refolding yield, using lysozyme as a model protein. This is done through a comparison of two reactor designs: a novel oscillatory flow reactor (OFR) (Mackley, Stonestreet, Roberts, & Ni, 1996) and a standard stirred-tank reactor (STR). The OFR configuration

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is of particular interest, as it is capable of simple scale-up without the mixing imperfections that typically characterise large stirred tanks (Harvey & Stonestreet, 2001). This configuration offers potential advantages for the refolding of proteins where yield is affected by mixing intensity.

2. Materials and methods

2.1. Materials

Lyophilised and dialysed hen egg-white lysozyme with a specific activity of approximately $70,000\ \mathrm{U\ mg^{-1}}$ was purchased from Fluka (Poole, UK). Guanidine hydrochloride (GuHCl), urea, oxidised glutathione (GSSG), Tris–HCl buffer and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (Poole, UK) and were ACS reagent grade. Dithiothreitol (DTT) was from Melford Laboratories (Chelsworth, UK). HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were also from Sigma.

2.2. Denaturation and reduction of lysozyme

Lysozyme approximately ($15\ \mathrm{mg\ ml^{-1}}$) was denatured in 4.7 M GuHCl, 8 M GuHCl, 8 or 10.2 M urea (all in 50 mM Tris–HCl, 1 mM EDTA, 32 mM DTT, pH 8.0 at 37°C) for 2 h. The concentration of denatured-reduced lysozyme (diluted into 0.1 M acetic acid) was determined at 280 nm, using an extinction coefficient of $2.37\ \mathrm{ml\ cm^{-1}\ mg^{-1}}$ (Clark, Hevehan, Szela, & Maachupalli-Reddy, 1998). Complete unfolding was checked using C_5 RP-HPLC as described subsequently, and was confirmed by activity assay.

2.3. RP-HPLC analysis of lysozyme

Native lysozyme concentration was measured using a C_5 reversed-phase column (5 μm, 300 Å, 150 mm × 4.6 mm, Jupiter, Phenomenex, Macclesfield, UK) on a high-performance liquid chromotography (HPLC) system comprising a X-Act 4-Channel Degassing Unit (Jour Research, Sweden), a 7725I Injection Valve (Rheodyne, USA), two HPLC 422 Pumps (Kontron Instruments, UK), a C030 HPLC Column Chiller/Heater (Torrey Pines Scientific, USA), a 2151 Variable Wavelength Detector (LKB, Sweden), and Chromeleon HPLC Management Software (Dionex, USA). A linear acetonitrile–water gradient with 0.1% (v/v) TFA [starting at 34% (v/v) acetonitrile, increasing at $1.2\%\ \mathrm{min^{-1}}$ to an end concentration of 46% (v/v)] was used to elute the samples, at a total solvent flowrate of $1\ \mathrm{ml\ min^{-1}}$. Absorbance was measured at 280 nm.

2.4. Activity assay

The loss of enzymatic activity following denaturation confirmed the absence of residual native protein. Enzymatic activity was measured by following the decrease in absorbance at 450 nm (Unicam UV1 Spectrophotometer, UK) of a cell suspension ($0.15\ \mathrm{mg\ ml^{-1}}$ *Micrococcus lysodeikticus*, 0.067 M sodium phosphate, pH 6.2). The denatured sample (100 μL, approximately $15\ \mathrm{mg\ ml^{-1}}$, in different concentrations of GuHCl or urea, 50 mM Tris–HCl, 1 mM EDTA, pH 8.0) was quenched and diluted in 5 ml of 0.1 M acetic acid. An aliquot of 20 μl diluted sample was added to 1 ml of cell suspension. The mixture was briefly mixed and, after 2 s, the absorbance was monitored for 40 s. No residual activity was observed for lysozyme denatured in 4.7, 6 or 8 M GuHCl, or 8 M or 10.2 M urea. A control experiment using native lysozyme quenched by the same procedure confirmed that it is not the quenching process that destroys biological activity.

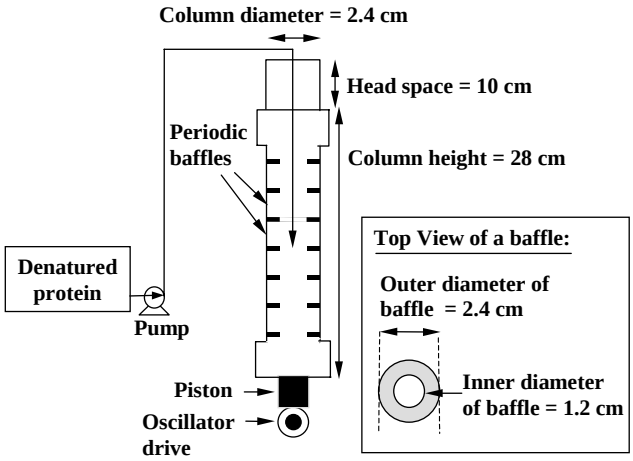

Fig. 1. A schematic diagram of the oscillatory flow reactor (OFR).

2.5. Oscillatory flow reactor

Fig. 1 shows a schematic diagram of the oscillatory flow reactor (OFR). The column is separated into eight cavities by seven annular baffles. A piston, driven by an oscillator drive, is used to oscillate the fluid within the column at a controllable frequency and amplitude. The denatured protein was fed through a single 1.6 mm needle that was placed within the fourth cavity of the column counting from the top. Mixing intensity is characterised by an oscillatory Reynolds number, Re_o, defined by Eq. (1),

$$Re_o = \frac{D\omega x_o}{\upsilon}, \tag{1}$$

where D is the tube diameter (m), ω is the angular frequency of the oscillator drive ($\mathrm{rad\ s^{-1}}$), x_o is the oscillatory amplitude measured from centre-to-peak (mm), and v is the kinematic viscosity ($\mathrm{m^2\ s^{-1}}$) (Mackley, 1991). A wide range of mixing intensities can be achieved simply by altering Re_o. At $Re_o \leqslant 400$, the flow pattern resembles axi-symmetric laminar flow. At $Re_o > 400$, the flow pattern is more turbulent-like (Mackley, 1991).

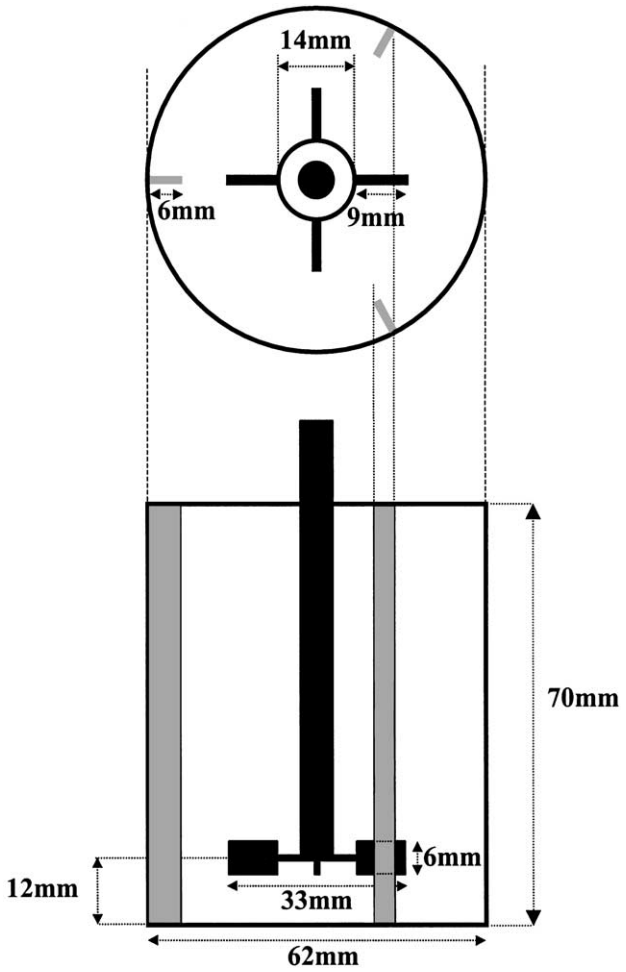

Fig. 2. A schematic diagram of the stirred-tank reactor (STR).

2.6. Fed-batch refolding in an OFR

Denatured-reduced lysozyme (14.0–15.5 mg ml^{-1} in the selected denaturing buffer) was fed into the OFR at a total flow rate of 0.09 ml min^{-1} for 120 min using a peristaltic pump (Watson Marlow 101 U/R), to give a final protein concentration of approximately 1.05 mg ml^{-1}. The initial volume of refolding buffer (4 mM GSSG, 50 mM Tris–HCl, 1 mM EDTA, pH 8, 20°C) was 140 ml. Refolding was conducted under intense (Re_o=1580, ω=22 rad^{-1}, x_o=3 mm) or mild ($Re_o = 250$, $\omega = 4.09$ rad^{-1}, $x_o = 1$ mm) oscillation. Upon completion of feeding, the solution was left for 3 h and oscillated at the selected mixing intensity. 1 ml samples were withdrawn hourly and quenched with 100 μl 10% TFA (v/v) for RP-HPLC analysis of refolding yield. All refolding experiments were duplicated.

2.7. Fed-batch refolding in a stirred-tank reactor

Refolding experiments conducted in a baffled stirred-tank reactor (STR), detailed in Fig. 2, provided a reference system for evaluating the performance of the OFR reactor. Denatured lysozyme (14.0–15.5 mg ml^{-1} in the selected denaturing buffer) was fed into the STR containing 140 ml of refold buffer (4 mM GSSG, 50 mM Tris–HCl, 1 mM EDTA, pH 8, 20°C), at a flow rate of 0.09 ml min^{-1} for 120 min using a peristaltic pump (Watson Marlow 101 U/R), to give a final protein concentration of approximately 1.05 mg ml^{-1}. The reaction mixture was agitated at 40, 180 and 350 rpm, to give Reynolds numbers (Re_T) of 726, 3267 and 6353, respectively. Upon completion of feeding, the solution was left for 3 h with mixing at the selected Re_T. 1 ml samples were withdrawn hourly and quenched with 100 μl of 10% (v/v) TFA for RP-HPLC analysis.

2.8. Batch refolding

Batch refolding of approximately 0.9 mg ml^{-1} of lysozyme was used to further examine the effects of denaturant. 100 μl of denatured lysozyme at 14.0–15.5 mg ml^{-1} was added into 1500 μl of refolding buffer (4 mM GSSG, 50 mM Tris–HCl, 1 mM EDTA, pH 8, 20°C) using a pipette, prior to rapid vortexing, in duplicate. Denatured lysozyme and refolding buffers were the same preparations used for OFR or STR experiments. Each batch reaction mixture was incubated for 3 h at room temperature and a 1 ml sample was then quenched with 100 μl of 10% (v/v) TFA for RP-HPLC analysis as described above.

3. Results and discussion

The fed-batch refolding results in the OFR using 8 M urea or 8 M GuHCl as the denaturant are illustrated in Fig. 3. Refolding yield following urea denaturation increased from 16% to 26% when oscillation intensity increased from $Re_o = 250$ to 1580, demonstrating a clear effect of mixing intensity. The enhanced initial dispersion of the denatured lysozyme, due to increased mixing intensity, resulted in a 63% relative increase in yield. Following GuHCl denaturation, refolding yields at both $Re_o = 250$ and 1580 were 32%, and no sensitivity to mixing intensity was observed. These results conclusively show that an interaction between the selected denaturant and the reactor mixing intensity can significantly affect refolding yield.

As illustrated in Fig. 4, refolding yield following urea denaturation increased from 18% to 24% when mixing intensity increased from $Re_T = 726$ to 6353 in the stirred-tank reactor (STR). This confirms that the observed dependence on mixing intensity is not reactor specific. A direct comparison of the data for both the OFR and the STR in Fig. 4 shows that, for this experiment, it is the extent of mixing and not the specific reactor design that controls renaturation yield. In these tests, the mixing intensity in the OFM cannot be directly compared with that in the STR, as equivalence rules are not yet available. The Reynolds numbers in each reactor were, therefore, chosen to give conditions of poor mixing (Re_T=726 and 250) or considerable turbulence (Re_T=6353

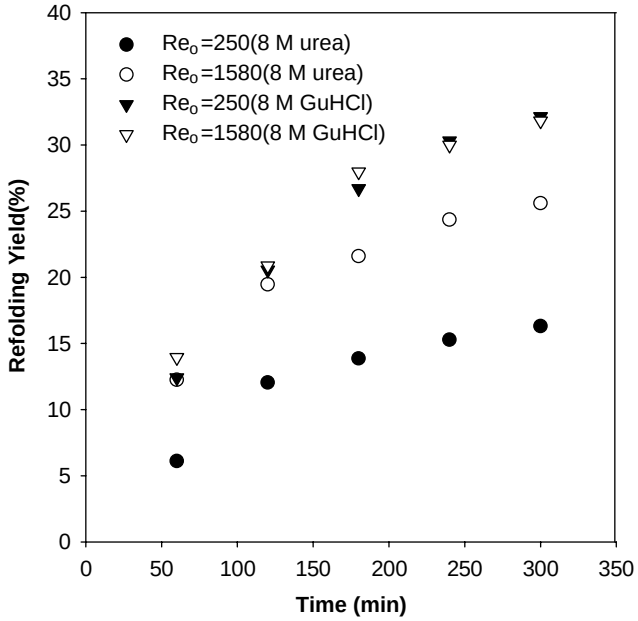

Fig. 3. Comparison of the refolding curves using 8 M urea-denatured lysozyme and 8 M GuHCl-denatured lysozyme at low mixing ($Re_o = 250$) and high mixing ($Re_o = 1580$).

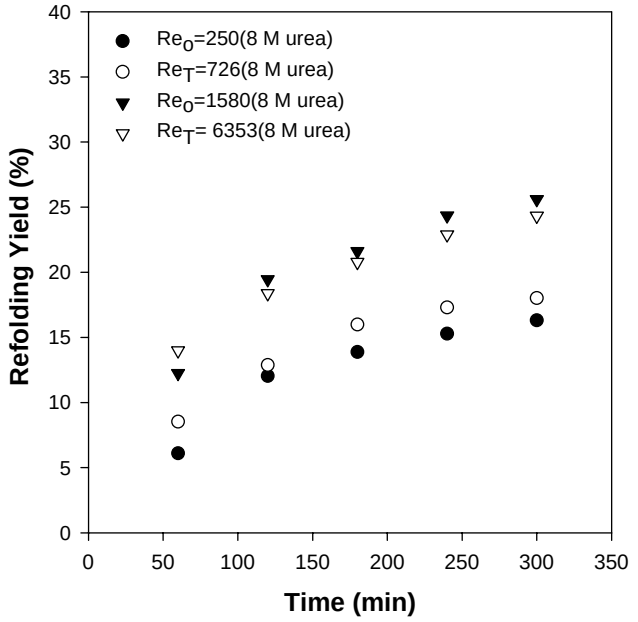

Fig. 4. Comparison of the refolding curves in both the OFR and STR at low mixing ($Re_o = 250$ for OFR and $Re_T = 726$ for STR) and high mixing ($Re_o = 1580$ for OFR and $Re_T = 6353$ for STR) following denaturation in 8 M urea.

and 1580). In these tests, a further increase in mixing intensity in each reactor did not increase renaturation yield. However, intermediate yields could be obtained by selecting an intermediate Reynolds number in either system.

Successful refolding in the OFR, and laboratory yields similar to the STR, confirms that the OFR is a valid refolding reactor. The importance of mixing intensity suggests that there are benefits in maintaining a uniform mixing environment on reactor scale-up. In this sense the OFR offers distinct advantages: it is claimed to be easier to scale than a STR if the intention is to obtain uniformity of mixing at process scale (Smith, 1999; Harvey & Stonestreet, 2001).

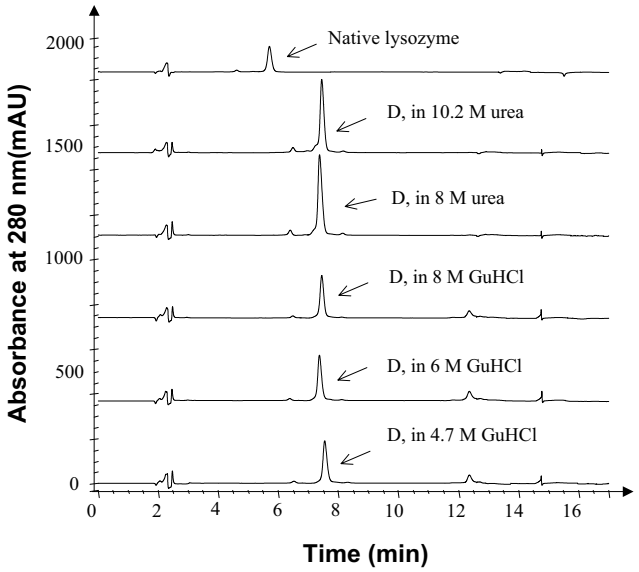

Fig. 5. HPLC chromatograms for native lysozyme, and denatured lysozyme (D) in 4.7, 6 and 8 M GuHCl, and 8 and 10.2 M urea.

It is well documented that GuHCl is a stronger denaturant than urea (Prakash, Loucheux, Scheufele, Gorbunoff, & Timasheff, 1981; West, Guise, & Chaudhuri, 1997). Denaturation curves for lysozyme using both urea and GuHCl suggest that GuHCl is 1.7 times more effective than urea (Greene & Pace, 1974). To test whether the mixing intensity effect we have observed is a function of the "denaturing power" rather than the precise denaturant, we compared refolding following denaturation in either 4.7 M GuHCl or 8 M urea (8 M = 1.7 × 4.7 M). Fig. 5 shows the RP-HPLC results for lysozyme denatured in 4.7 M GuHCl, confirming that unfolding is complete. All other peaks of denatured lysozyme (in 8 M GuHCl, 8 and 10.2 M urea), and the lysozyme native peak, are also illustrated in Fig. 5. As shown in Fig. 6, the refolding results obtained following denaturation in 4.7 M GuHCl or 8 M urea are comparable at three different mixing intensities ($Re_T = 726$, 3267 and 6353). This demonstrates that the observed effect of mixing intensity depends strongly on the effectiveness of the denaturant and not the specific type of denaturant. This interaction between "denaturing power" and mixing intensity has not been previously identified in the literature, and indeed the effect of mixing has only been superficially investigated.

To further compare the effect of different strengths of denaturant on the refolding yield, batch refolding tests were conducted using lysozyme denatured in 4.7 M GuHCl, 6 M GuHCl and 10.2 M urea. Table 1 lists the results of selected

Table 1
Batch refolding results for lysozyme denatured under different conditions (after dilution, the final refolding buffer contained 1 mg ml^{-1} of lysozyme, 3.75 mM GSSG, 1 mM EDTA, 50 mM Tris–HCl, and different denaturant concentrations)

Initial denaturant concentration	Denaturant concentration in the refolding buffer before dilution	Final concentration of denaturant in the refolding buffer after dilution	Refolding yield (%)
8 M urea	0	0.5 M urea	6.7 ± 0.8
10.2 M urea	0	0.64 M urea	9.3 ± 0.3
4.7 M GuHCl	0	0.29 M GuHCl	5.5 ± 0.7
6 M GuHCl	0	0.38 M GuHCl	8.2 ± 0.5
8 M GuHCl	0	0.5 M GuHCl	16.2 ± 1.3
4.7 M GuHCl	0.22 M GuHCl	0.5 M GuHCl	19.2 ± 0.1
8 M GuHCl	0.22 M GuHCl	0.71 M GuHCl	32.0 ± 0.1

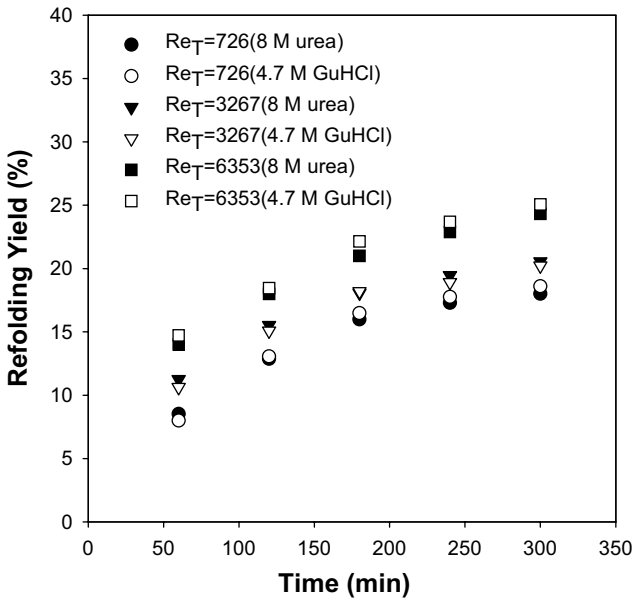

Fig. 6. Comparison of the refolding curves obtained at three mixing intensities ($Re_T = 726$, 3267, and 6353) when 8 M urea or 4.7 M GuHCl were used as the denaturant.

batch tests. The comparable "denaturing power" of 8 M urea and 4.7 M GuHCl has resulted in similar yields of 6.7% and 5.5%. The refolding yields following denaturation with 10.2 M urea and 6 M GuHCl are also similar, especially considering that the 1.7 × factor is only approximate. A high yield of 16% was achieved when dilution occurred from the strongest denaturing conditions (8 M GuHCl). It has been widely reported that the inclusion of folding enhancers in the refolding buffer can inhibit aggregation and thus increase refolding yield (Clark & Georgiou, 1991; Maeda, Koga, Yamada, Ueda, & Imoto, 1995). It is also well known that chaotrope generally acts as a folding enhancer, and it has been proposed that this is due to shielding of the hydrophobic patches on partially folded protein molecules (Jaenicke & Rudolph, 1989). For lysozyme, the refolding yield in tests where chaotrope is present (Raman, Ramakrishna, & Rao, 1996; Yasuda, Murakami, Sowa, Ogino, & Ishikawa, 1998; Clark et al., 1998) are consistently higher than when chaotrope is not included in the refolding buffer (Saxena & Wetlaufer, 1970; Goldberg et al., 1991). It is also known that inclusion of arginine in refolding buffer inhibits aggregation through a chaotrope-like binding to exposed hydrophobic regions (Jaenicke & Rudolph, 1989). The trend of increased yield with increasing residual chaotrope concentration, reported in Table 1, is, therefore, unsurprising. To further examine this, we added chaotrope into the refolding buffer before batch addition of denatured protein. When 0.22 M GuHCl was included in the refolding buffer, to give a final concentration of 0.5 M, yield increased to $19.2 \pm 0.1\%$ following denaturation in 4.7 M GuHCl. This compares with $16.2 \pm 1.3\%$ following denaturation in 8 M GuHCl, at the same final GuHCl concentration. The inclusion of chaotrope in the refolding buffer does indeed enhance the refolding yield.

However, if chaotrope acts only as a folding enhancer, then one might not expect the strong dependence on mixing intensity observed following urea denaturation. The time-variant concentrations during dilution may also be important (i.e., it is not just the end-point protein and chaotrope concentrations that determines yield, but also their rate of change). To test this hypothesis for urea, we conducted two sets of parallel batch tests in small tubes. In the first, denatured protein was rapidly mixed into the refolding buffer following denaturation in 8 M urea. A refolding yield of $6.0 \pm 0.3\%$ was obtained, consistent with the batch result reported in Table 1. In the second set of tests, the denser solution of denatured protein was carefully injected into the bottom of the refolding buffer, without mixing, and the solution was allowed to homogenise by diffusion. A low and variable refolding yield of $3.4 \pm 1.5\%$ was obtained, confirming a dependence on the "path" of dilution or dispersion.

It is, therefore, probable that the chaotrope acts in two distinct ways. Chaotrope in the denatured protein stream may shield intermediates during the initial dispersion phase, thus inhibiting aggregation. A high denaturing power during dispersion preserves protein solubility and slows both the refolding and aggregation reactions, ensuring that the

molecules remain monomeric until fully dispersed. Complete dispersion reduces the effective protein concentration, subsequently promoting unimolecular refolding in preference to higher-order aggregation. Following this initial dispersion phase, residual chaotrope in the refolding buffer inhibits aggregation of the dispersed but partially folded intermediates. Under this hypothesis there is, for lysozyme, a clear benefit in maintaining a high denaturing power in both the denatured protein stream and in the refolding buffer. The final row in Table 1 confirms this assertion.

Considerable work remains to generalise the current findings. In some cases, the inclusion of chaotrope in the refolding buffer can be detrimental. For example, the refolding of heterodimeric platelet-derived growth factor (Muller & Rinas, 1999) and bone morphogenetic protein-2 (Biron, 1999) was either inhibited or retarded in the presence of even low concentrations of denaturant. The results of mixing studies will, therefore, be a complex function of the refolding energy landscape, and for most proteins this is poorly defined. Many other factors (e.g., protein concentration, redox potential, pH) will also affect aggregation rate and thus final yield. The complex interaction of these parameters will, in all likelihood, preclude the definition of a universally applicable optimal mixing intensity.

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