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Initial Protein Concentration and Residual Denaturant Concentration Strongly Affect the Batch Refolding of Hen Egg White Lysozyme

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Abstract The effects of several variables on the refolding of hen egg white lysozyme have been studied. Lysozyme was denatured in both urea, and guanidine hydrochloride (GuHCl), and batch refolded by dilution (100 to 1000 fold) into 0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, 3 mM reduced glutathione and 0.3 mM oxidised glutathione. Refolding was found to be sensitive to temperature, with the highest refolding yield obtained at 50°C. The apparent activation energy for lysozyme refolding was found to be 56 kJ/mol. Refolding by dilution results in low concentrations of both denaturant and reducing agent species. It was found that the residual concentrations obtained during dilution (100-fold dilution: [GuHCl] = 0.06 mM, [DTT] = 0.15 mM) were significant and could inhibit lysozyme refolding. This study has also shown that the initial protein concentration (1 - 10 mg/mL) that is refolded is an important parameter. In the presence of residual GuHCl and DTT, higher refolding yields were obtained when starting from higher initial lysozyme concentrations. This trend was reversed when residual denaturant components were removed from the refolding buffer.

Keywords: protein refolding, lysozyme, GuHCl, urea, acetic acid

INTRODUCTION

The production of recombinant proteins via inclusion bodies is likely to remain an important manufacturing route for the foreseeable future. Protein activity is recovered by dissolving the inclusion bodies in a strong denaturant, and initiating refolding by removing the denaturant [1,2]. Currently, batch dilution is the preferred method for refolding most recombinant proteins due to its simplicity. However, dilution is an inefficient process, with protein activity recoveries less than 20% being common. Protein refolding is a first order process [3], however, the low yields result from loss of the majority of protein via a second order process involving non-specific hydrophobic interactions between folding intermediates. This results in insoluble protein aggregates [4].

Many methods of improving refolding yields have been studied. These include washing the inclusion bodies to remove any loosely bound contaminants that might interfere with refolding [5,6], and addition of chemicals to the refolding buffer, for example L-arginine [7], and polyethylene glycol [8]. Such additives enhance refolding yields by reducing non-productive intra- and inter-molecular reactions. Thereby reducing aggregation and increasing the yield of refolded protein.

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Alternative ways of physically removing the denaturant from the solubilised inclusion bodies have been reported. For example, dialysis and diafiltration have been used to reduce the denaturant concentration and at the same time supply the refolding solution to the denatured protein. Dialysis has been used to refold tryptophanase [9] and bovine growth hormone [10], whereas prorennin [10] and carbonic anhydrase [11] have been refolded by diafiltration. The use of gel filtration chromatography for refolding has also been attempted [12,13]. This prevents individual refolding polypeptides from interacting by physical exclusion of the molecules in the gel pores.

The solvent conditions during refolding are extremely important. It is necessary for the denaturant concentration to be reduced to a level that allows the protein to refold but the residual denaturant concentration governs which intermediate species proliferate. It is important however to remember that due to the equilibrium existing between the native and the denatured states that an increase in denaturant concentration also increases the number of denatured molecules in the system. These molecules may then form aggregates which are insoluble. The concentration of denaturant at which aggregating species predominate is different for different proteins and denaturants. The literature indicates that increasing either the initial or final concentration of protein in a refolding system decreases the obtained yield [7,14,15]. The decrease in yield is again due to unfavourable intermolecular reactions which lead to ag-

gregation. As the concentration of refolding protein increases these interactions become prevalent and the observed yield of refolded protein is reduced. Thus, protein concentrations are kept low during folding to prevent aggregation. Typically, to achieve high yields denatured protein concentrations of less than 0.01 mg/mL are used.

Despite the reports of the influence of solvent conditions on refolding the literature available on the effect of environmental parameters on refolding is incomplete and is in places contradictory. The effect of temperature and pH have been studied previously but only over limited ranges. The effect of the ratio of reducing and oxidising thiol groups in the refolding buffer has been studied and optimised. However, the effect of residual denaturant in the refolding buffer on the refolding yield however has not been widely reported. The importance of the effect of the lysozyme concentration in the reduced form, or in other words the dilution factor, prior to refolding on the yield of refolded protein has generally been overlooked. We report here a systematic study on the effects of denaturant type, residual denaturant concentration and dilution factor on protein refolding yields. These are variables that have not been widely studied, but have a significant effect on protein refolding yields.

For this study we have used hen egg white lysozyme as the model protein. This enzyme has been extensively used for protein refolding studies. It is a single subunit protein containing 129 residues with a molecular weight of 14,000 daltons. Lysozyme is an extremely stable enzyme and is cross-linked by four disulphide bonds. The refolding of non-reduced and reduced lysozyme has been the subject of several studies. The method of refolding has generally been to reduce the concentration of denaturant and reducing agent by dilution into a refolding buffer. The refolding of non-reduced lysozyme is facile and yields of 90% at concentrations greater than 0.5 mg/mL are possible [16]. Using circular dichroism it has been shown that during the refolding of non-reduced lysozyme that 80% of the native structure is regained in less than 4 ms [17]. The refolding of reduced lysozyme is more complicated and involves the oxidation of the four disulphide bonds. Epstein and Goldberger [18] showed that lysozyme refolding was dependent on the protein concentration, pH, thiol concentration, and temperature. The yield of refolded protein increased from pH 7-8.5 and a higher yield was achieved at 38°C than at 25°C. Saxena and Wetlaufer [19] found that the optimal conditions for refolding with respect to thiol concentration were 5 mM reduced glutathione and 0.5 mM oxidised glutathione.

MATERIALS AND METHODS

Materials

Hen egg white lysozyme (EC 3.2.1.17; 52,000 units/mg), *Micrococcus lysodeikticus* dried cells, oxidised and reduced glutathione (GSSG and GSH), guanidine hydrogen chloride (GuHCl), urea, dithiothreitol (DTT), Tris-HCl, potassium di-hydrogen orthophosphate, EDTA, and nitric acid were obtained from Sigma Chemicals. (Poole, Dorset). All chemicals were of analytical grade.

Experimental Methods

Preparation of Denatured Lysozyme

Lysozyme was denatured using methods adapted from those of Goldberg *et al.* [15]. Three solutions of denatured lysozyme were used. Solutions of 10 mg/mL lysozyme in either 6 M GuHCl (with 0.15 M DTT and 0.1 M Tris-HCl pH 8.2) or 8 M urea (with 0.15 M DTT and 0.1 M Tris-HCl pH 8.2) were prepared and then left to incubate at room temperature for two hours before use. In addition, the GuHCl-denatured lysozyme solution was dialysed against 0.1 M acetic acid and then micro-centrifuged to remove any aggregated material.

Renaturation of Lysozyme

In general lysozyme renaturation was initiated by diluting the 10 mg/mL solution of denatured reduced lysozyme between 100-1000 fold with refolding buffer to give a final concentration between 0.01 to 0.2 mg/mL in the refolding buffer. The refolding buffer comprised 0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, 3 mM reduced glutathione and 0.3 mM oxidised glutathione [15]. The renaturation solution was incubated in a water bath at 40°C unless stated otherwise.

Analytical Techniques

Lysozyme concentrations were determined spectrophotometrically (Series 3000, Cecil Instruments, Cambridge, UK) at 280 nm, using an extinction coefficient of 2.63 units mL/mg for native lysozyme and 2.37 units mL/mg for denatured lysozyme, with a 1 cm path length [20]. Lysozyme activity was determined at 25°C by following the decrease in absorbance at 450 nm of a 0.25 mg/mL suspension of *Micrococcus lysodeikticus* in 60 mM potassium phosphate buffer, pH 6.2. 100 μL of solution from the refolding buffer was added to 900 μL of *Micrococcus* suspension [21]. A decrease in absorbance of 0.0026 units per minute corresponded to one unit of activity. Lysozyme activities are reported as a percentage of the activity expected from the same molar concentration of native lysozyme. This percentage is termed the yield of refolding and is equivalent to the specific activity of the refolded protein divided by the specific activity of native lysozyme under identical conditions [19]. The increase in turbidity caused by aggregation was measured at 450 nm. In order to assess whether non-active protein had aggregated, the aggregates in 1 mL of solution were collected by micro-centrifugation, re-dissolved in 1 mL of 6 M GuHCl (0.1 M Tris-HCl, pH 8.2) denaturant and the concentration of the dissolved protein measured. The results presented here are mean of three experiments. The error between experiments was no greater than 5%.

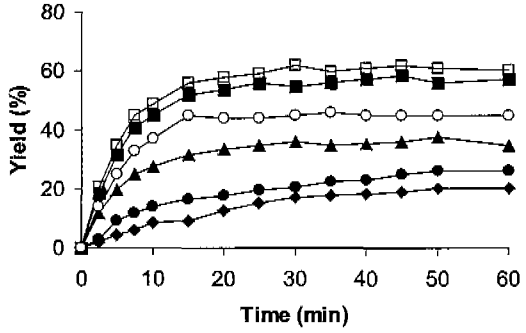

Fig. 1. The effect of temperature (°C) on the refolding of lysozyme: 4 (◆), 10 (●), 20 (▲), 40 (■), 50 (□), 60 (○).

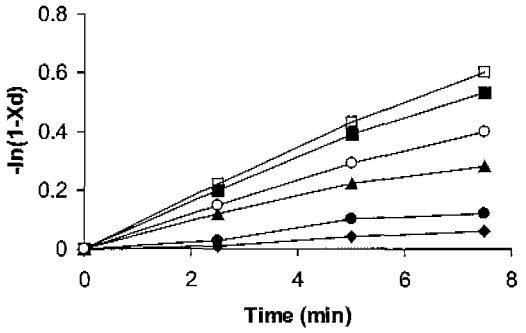

Fig. 2. Calculation of the initial rates of refolding at different temperatures (°C): 4 (◆), 10 (●), 20 (▲), 40 (■), 50 (□), 60 (○).

RESULTS AND DISCUSSION

Temperature Dependence of Lysozyme Refolding

Lysozyme, denatured with GuHCl and dialysed against acetic acid, was refolded by dilution in refolding buffer to give a final concentration of 0.01 mg/mL. Fig. 1 shows the effect of temperature on the refolding yield. An increase in temperature from 4°C to 50°C was found to increase the yield of refolded protein from 20% to 64%, respectively. The refolding yield decreases again at 60°C. No aggregates were detected for all temperatures studied.

The Arrhenius law may be used to describe the results in Fig. 1.

$$k = k_0 e^{\frac{-E}{RT}} \tag{1}$$

where k is the specific rate constant (s^{-1}), R is the gas constant (J mol^{-1} K^{-1})=8.31, k_0 is the frequency factor (s^{-1}), T is temperature (K) and E is the activation energy (J/mol). Plotting ln k versus $1/T$ gives ln k_0 as the intercept and $-E/R$ as the gradient.

In order to determine the activation energy the rate constant for each temperature was calculated. It was assumed that k is a function of temperature only and that refolding is a first order reaction. For a first order reaction, the rate, r_A, is given by:

$$r_A = C_{D0}\frac{dX_D}{dt} = -k\ C_D \tag{2}$$

Where r_A is the rate of refolding mg mL^{-1} s^{-1}; X_D is the conversion of denatured protein; C_{D0} is the initial concentration of denatured protein (mg/mL) and C_D is the concentration of denatured protein at time t (mg/mL)

The conversion of denatured protein, X_D, at any given time can be defined as:

$$X_D = 1 - \frac{C_D}{C_0} \tag{3}$$

Thus, by plotting ln(1-X_D) against t for renaturation experiments at different temperatures the values of k for each temperature can be calculated from the initial rates of reaction (Fig. 2).

The activation energy for lysozyme refolding was calculated to be 56 kJ/mol (r^2 = 0.98). The data at 50°C and 60°C were not used in the calculation of the activation energy. These points did not fit into the linear plot for the Arrhenius equation (data not shown). This is not unexpected as at such temperatures it is likely that the protein is thermally denatured [22].

Privalov [23] showed that the free energy of refolding for a small globular protein can be expected to be approximately 50 kJ/mol. However, the activation energy in our study is not a direct representation of the activation or free energy of refolding. The concentration of lysozyme in the refolding buffer for these experiments was 0.01 mg/mL. Even at this low concentration the refolding reaction is subject to a competitive aggregation reaction. The value calculated for the activation energy for refolding can be considered as an apparent activation energy which takes into account the effect of temperature on the aggregation process as well as refolding.

The majority of refolding processes are performed in the range 0-40°C with 20-25°C most commonly used. At temperatures well above this range the efficiency of folding of the proteins studied is decreased due to thermal denaturation [24,25]. However, optimal temperatures for refolding have to be determined on a protein-specific basis. Studies of several proteins including rhodanese [26], the dimeric form of Rubisco [27], F_{ab} fragments [28] and the phage P22 tailspike protein [29] have shown that reactivation increases at low temperatures with a maximum at around 10°C. The increase in renaturation at lower temperatures is related to the reduction in the rate of aggregation allowing more protein molecules to reach the native state [8].

It is thought that temperature directly affects the endothermic hydrophobic interactions which are thought to be the main driving force for refolding [30]. Therefore an increase in temperature should lead to an increase in the rate of refolding. However an increase in temperature may also lead to an increase in denatured protein concentration and therefore an increase in aggregation. For each individual protein and given set of

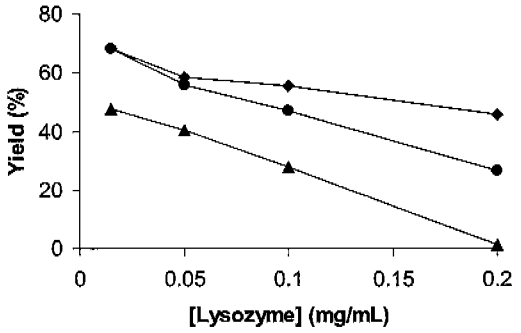

Fig. 3. The effect of type of denaturant on the refolding yield of lysozyme. Acetic acid (◆), guanidine hydrochloride (●) and urea (▲).

conditions a balance between these two phenomena must be found. If the thermally denatured molecules could be stabilised then refolding would be possible at higher temperatures. In the case of Rubisco it was found that efficient renaturation could be achieved at temperatures up to 37°C when refolded in the presence of chaperonins [31].

Effect of Denaturant Type

The type of denaturant used to unfold lysozyme has a significant effect on the refolding yield. We studied the effects of GuHCl, urea and acetic acid (Fig. 3). In all cases as the concentration of lysozyme in the refolding buffer is increased the yield of refolded native lysozyme is reduced. The maximum refolding yield achieved is similar for both acetic acid and from GuHCl (68%). The maximum yield obtained for the refolding of lysozyme from urea was 49%. When refolding from urea and from GuHCl there is a significant decrease in the observed yield as the concentration of the protein in the buffer is increased. In the case of urea the yield falls from 49% to zero and over the same range the yield of refolding from GuHC1 falls from 68% to 27%. The loss in yield is lower for the refolding from acetic acid the yield falls from 68% to 50%.

The difference in the yield of refolded lysozyme at low lysozyme concentration when refolding from different denaturants is thought to be due to the effect of the low residual concentration of the denaturants in the refolding buffer and the state of the denatured molecule prior to refolding. Denatured, reduced lysozyme in 0.1 M acetic acid has a more compact structure than denatured reduced lysozyme in either 6 M GuHCl or 8 M urea [19]. Therefore when the denatured reduced lysozyme in acetic acid is diluted into refolding buffer it already has a more stable structure than the unfolded lysozyme in the other denaturants. This in turn leads to higher refolding yields. It is proposed that the refolding yield of GuHCl is higher than the refolding yield of urea because of the ionic nature of the GuHCl. At low concentrations this will stabilise the refolding intermediates formed. Matsubara *et al.* [32] showed that the kinetics of lysozyme refolding were inhibited by increasing concentrations of urea in the refolding buffer.

The decrease in yield observed with increasing lysozyme concentration in the refolding buffer is well documented and results from competitive protein-protein aggregation. The reason for the greater loss of activity when refolding from GuHCl and urea is likely to be a result of the accumulating concentrations of denaturant and reducing agent in the refolding buffer. For the refolding of lysozyme from GuHCl it has been shown that the protein refolds to a compact native like state (molten globule) within 4 ms [17]. Fluorescence experiments on lysozyme [33] suggest that this structure is less compact in increasing concentrations of GuHCl in the refolding buffer, and therefore the molecule is less stable and more likely to aggregate.

Effect of Residual GuHCl and DTT Concentrations on the Refolding of Lysozyme

When refolding from typical denaturing conditions of 6 M GuHCl and 0.15 M DTT into refolding buffer there will be a residual concentration of denaturant and reducing agent present in the refolding buffer. For example a 100-fold dilution will result in a solution of 0.06 M GuHCl and 1.5 mM DTT. This residual concentration may have a significant effect on the refolding of lysozyme. To investigate this, lysozyme was denatured and dialysed into acetic acid. GuHCl was then added to the renaturation buffer to give a desired concentration and in a separate experiment DTT was similarly added to the renaturation buffer. Lysozyme was refolded by dilution into the appropriate buffer to a final concentration of 0.01 mg/mL.

Fig. 4 shows that the refolding of lysozyme is extremely sensitive to the residual concentration of denaturant in the refolding buffer. At 0.02 M GuHCl refolding is strongly inhibited and at 0.04 M there is very little regain of activity. In all experiments no aggregates were detected at 450 nm. Fig. 5 shows the effect of residual DTT on refolding. Refolding is highest at low concentrations of DTT and falls rapidly to zero reactivation at concentrations above 0.6 mM. This is probably due to the shift in the redox potential of the system. As the concentration of DTT is increased the refolding buffer becomes a more reducing environment, disulphide bonds will not reform properly under these conditions and therefore less activity is recovered.

As the residual concentration of GuHCl is increased both the ionic strength and chaotropic nature of the molecule are increased. The concentration of denaturant in the refolding buffer determines which refolding intermediates proliferate. During denaturation GuHCl binds to hydrophobic residues and exposes the hydrophobic core of the protein [34]. These combined effects may lead to an increase in unproductive intermolecular associations which would explain the decrease in yield observed. However, no aggregates could be detected at 450 nm, even at 0.1 M GuHCl when less than a 10%

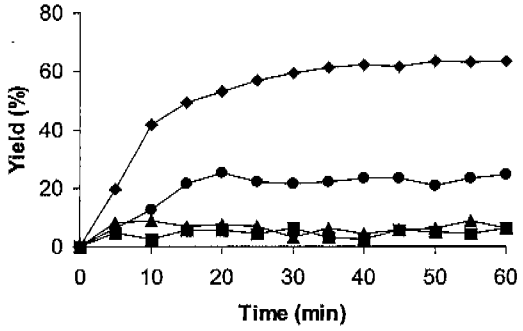

Fig. 4. The effect of residual GuHCl concentration on the refolding of lysozyme: 0.01 M (◆), 0.02 M (●), 0.04 M (▲) and 0.1 M (■).

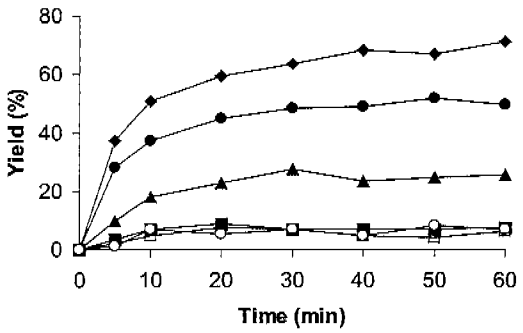

Fig. 5. The effect of DTT concentration (mM) on the refolding of lysozyme: 0.15 (◆), 0.3 (●), 0.45 (▲), 0.6 (■), 1.2 (□), 2.4 (○).

yield is achieved, aggregation does not increase significantly, the increase in ionic strength of the refolding buffer is not likely to be responsible for the observed loss in yield.

A more likely explanation for the observed loss in yield is the effect of the denaturant on the active site of the protein. The active site of an enzyme is extremely sensitive and during the denaturation of a number of enzymes it has been observed that inactivation occurs at lower concentration of denaturant than denaturation [35]. Enzymes which are inactivated prior to unfolding include creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, ribonuclease A and lactic dehydrogenase [36]. The low concentration of denaturant may well disrupt the active site of lysozyme enough to reduce the yield of active protein. The active site of the lysozyme is buried in an ellipsoidal cleft close to the hydrophobic core of the protein. The active site consists primarily of two amino acids, Glu 35 and Asp 52. Asp is negatively charged and would be expected to interact with the positively charged Gdm^+ ion. This association and the association of Gdm^+ with hydrophobic residues close to the active site would explain the decrease in yield as the residual concentration of GuHCl is increased. This is supported by the work of Denton *et al.* [33] who showed that increasing the concentration of GuHCl in the refolding buffer increased the size of lysozyme refolding intermediates. This is consistent with the observation that although there is a decrease in yield there is no observed increase in aggregation.

Adding DTT to the system shifts the equilibrium of reducing and oxidising agent. In this experiment a ratio of 3 mM reduced glutathione to 0.3 mM oxidised glutathione is used. There is practically no refolding above 0.6 mM DTT. This approximates to a change in the molar redox ratio of reduced and oxidised thiol in the system from 10:1 to 3:1. The loss in activity is consistent with the results of [19] who found that at a concentration of 3 mM reduced glutathione and a redox ratio of three to one that no activity was regained.

Although the effect of DTT on the refolding of proteins has not been reported in detail the effect of other thiol agents such as reduced and oxidised glutathione (GSH and GSSG) has been studied. Saxena and Wetlaufer [19] found that regeneration of active lysozyme is highest at a 10:1 molar ratio of reduced glutathione to oxidised glutathione and when the concentration of reduced glutathione is 5×10^{-3} M. It may seem odd at first that optimal refolding occurs under reducing conditions. However *in vivo* folding generally occurs under reducing conditions. Protein disulphides are generally more stable than non-cyclic thiol groups. A reducing system containing both reducing and oxidising agents allows misformed unstable disulphides to break and reform properly. Once formed correctly the reducing potential of the system is not high enough to reduce the stable bond.

Several studies have examined the effect of residual (*i.e.* low) denaturant concentration on refolding. Damodaran [37] found that the refolding of bovine serum albumin was inhibited when 2.0 M urea was present in the refolding buffer. It has been shown that residual denaturant concentration has a detrimental effect on the recovery of activity during the refolding of lactic dehydrogenase [38]. CD and fluorescence measurements have been used to show that low concentrations of GuHCl expose apolar residues and reduce the yield of refolded rhodanese [34].

When refolding proteins by dilution the level of denaturant in the refolding buffer has to be reduced to a level which allows refolding to occur. The optimum concentration of GuHCl in the refolding buffer is protein specific and depends on the refolding intermediates formed at that concentration. For the refolding of lysozyme it has been shown that refolding is optimal at low concentrations of GuHCl. At 0.01 mg/mL the loss in activity is not due to an increase in aggregation but is probably due to inactivation of the protein. These results support the theory that the observed decrease in activity when refolding protein from different concentrations of denatured reduced protein is due to increasing denaturant concentration. They also suggest that for the refolding of lysozyme that the denaturant should be removed from the refolding system completely.

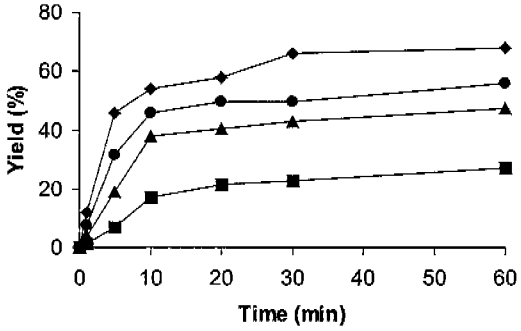

Fig. 6. The effect of final protein concentration (mg/mL) on the yield of refolded lysozyme: 0.015 (◆), 0.05 (●), 0.1 (▲), 0.2 (■).

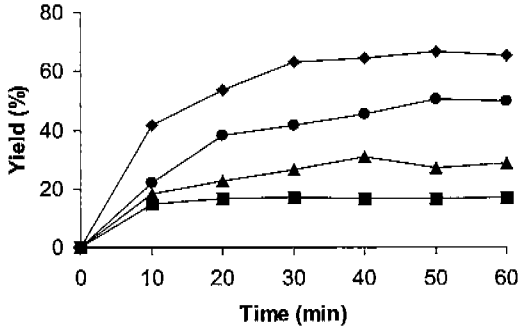

Fig. 7. The effect of initial protein concentration (mg/mL) on the refolding of lysozyme: 1 (■), 2 (▲), 5 (●), 10 (◆).

Effect of Final Protein Concentration on Lysozyme Refolding

Lysozyme was refolded by dilution with refolding buffer to give a final enzyme concentration between 0.015 to 0.2 mg/mL. The effect of increasing the lysozyme concentration on the yield of active protein can be seen in Fig. 6. As the amount of denatured reduced lysozyme added to the refolding buffer is increased from 0.015 to 0.2 mg/mL the observed yield of active protein is decreased. The yield falls from 68% at 0.015 mg/mL to 28% at 0.2 mg/mL. The decrease in yield can be attributed to aggregation of folding molecules. The turbidity of the refolding buffer increases from zero at 0.015 mg/mL to approximately 0.75 units (450 nm) at 0.2 mg/mL. The protein precipitates as it aggregates and at 0.2 mg/mL the white aggregates can be clearly seen. Performing a mass balance on the protein added to the system it was calculated that any non-active protein had aggregated (*e.g.* when refolding at 0.2 mg/mL a yield of 28% is obtained. The concentration of dissolved aggregates was approximately 0.14 mg/mL). Protein refolding is initiated by the removal of the denaturant, usually by dilution, which initiates the collapse of the unfolded molecule and the formation of secondary/early tertiary structure. The collapse of the molecule is thought to occur on a milli-second time-scale [17]. It is during this time that hydrophobic residues will become exposed and the protein will be most susceptible to aggregation [9,34]. If the protein concentration is high then non-productive reactions will predominate as hydrophobic regions are exposed and interact with exposed regions from other protein molecules. In the case of proteins with disulphide bonds, incorrect disulphide bond formation may also lead to aggregation [39]. In dilute systems the aggregation reaction becomes diffusion limited and the refolding reaction predominates. To obtain a reasonable yield (*i.e.* greater than 25%) refolding often has to be carried out at concentrations of less then 0.01 mg/mL [40].

Effect of Initial Protein Concentration on Lysozyme Refolding

The effect of the initial protein concentration on refolding yield was determined by diluting denatured lysozyme from a starting concentration between 1 to 10 mg/mL to give a final concentration of 0.01 mg/mL in refolding buffer. Fig. 7 shows the effect of the initial concentration on the refolding yield. As the starting protein concentration was increased from 1 to 10 mg/mL the observed yield increased from 17% to 65%. One would expect that as the initial concentration of the protein is decreased, the yield should increase as the intermolecular reactions are reduced. However, as the protein concentration is decreased from 10 to 1 mg/mL, the dilution factor is decreased ten fold from 1 in 1000 to 1 in 100. Consequently, the residual concentration of GuHCl and DTT in the refolding buffer increases from 6 mM and 0.15 mM to 60 mM and 1.5 mM, respectively. Thus at the lowest protein concentration, the residual denaturant concentration is highest and at inhibitory levels. These results suggest that both the residual denaturant concentration and the redox potential of the refolding buffer have a detrimental effect on the observed yield of refolding lysozyme.

To investigate further the effect of the initial protein concentration without the detrimental effects of GuHCl and DTT being present the previous experiment was repeated, except the stock denatured reduced lysozyme was dialysed into 0.1 M acetic acid. This solution was diluted between 100 and 1000 times into refolding buffer at 40°C to give a final concentration of 0.01 mg/mL of lysozyme. The pH change due to the addition of different amounts of acetic acid was measured and found to be negligible. The results are shown in Fig. 8 and are the reverse of the trend seen in Fig. 7. As the initial concentration of lysozyme is decreased the observed yield is increased from 68% to 95%. This result confirms the inhibitory effect of residual denaturant and reducing agent in the refolding buffer.

A further experiment was carried out for different final concentrations of lysozyme. Fig. 9 shows how the initial protein concentration affects the observed yield

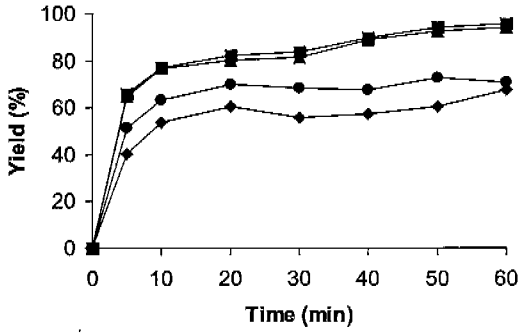

Fig. 8. The effect of initial protein concentration (mg/mL), in acetic acid, on the refolding of lysozyme: 1 (■), 2 (▲), 5 (●), 10 (◆).

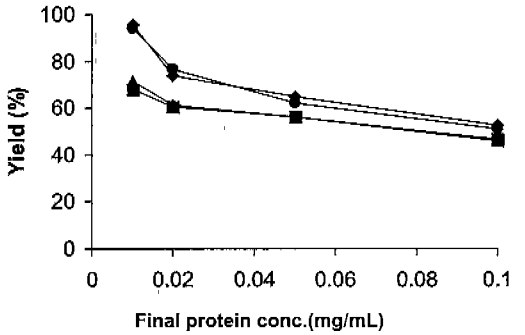

Fig. 9. The effect of both the initial protein concentration and the final protein concentration on the refolding of lysozyme. Initial concentration (mg/mL): 1 (◆), 2 (●), 5 (▲) and 10 (■).

of refolded protein at different final concentrations of protein. The initial protein concentration has the greatest effect on refolding when the final concentration of protein is low (0.01 mg/mL). The effect on refolding yield becomes independent of the initial protein concentration as the final protein concentration is increased. At a final concentration of 0.1 mg/mL there is less than 10% difference in the final yield observed between all starting concentrations.

The reason for the reduced difference in the effect of dilution at different concentrations of refolding lysozyme is probably due to the increased numbers of interactions between folding intermediates at high initial concentration of protein. This explanation is schematically illustrated in Fig. 10. The diagram indicates the refolding yield obtained for a given final lysozyme concentration. Letters A→D indicate the possible starting concentrations of denatured lysozyme prior to refolding. Numbers 1→4 indicate the final concentration of refolded lysozyme in the refolding buffer after dilution. The diagram shows that when refolding from a high (10 mg/mL) to a moderate (1 mg/mL) starting concentration of denatured lysozyme, that the yield is low and aggregation dominates. The time taken for the

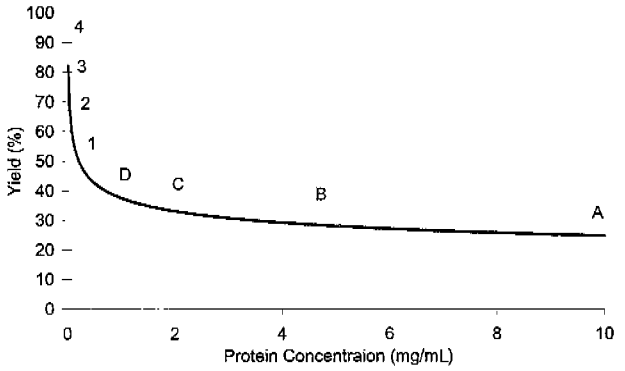

Fig. 10. Schematic relationship between refolding yield and initial and final protein concentrations.

denatured protein to move from concentration A to 4, *i.e.* to be dispersed homogeneously and reduce intermolecular interactions, is significant and aggregation is likely. If, however, the initial denatured protein concentration is lower at position D (<1 mg/mL), then the effect of dispersion time is reduced and the probability of protein-protein interactions becomes lower. The time taken to reach a concentration where refolding dominates and aggregation is minimal is less than when starting from a concentration of 10 mg/mL, *i.e.* position A. Therefore a higher yield will be obtained when refolding from dilute denatured protein than when refolding from concentrated denatured protein.

The reason for the dilution effect being less pronounced when refolding at higher final protein concentration when the final yield is low can be similarly explained. When refolding from moderate to high lysozyme concentrations (*i.e.* > 1 mg/mL) to position 1 (0.1 mg/mL) the yield is always low as aggregation is extensive. The difference in time taken to disperse a 1 mg/mL or a 10 mg/mL solution, *i.e.* moving from position A to 1 or from D to 1, is insignificant as there is a high probability of aggregation.

When the effect of protein concentration on refolding is discussed, it is usually the final concentration of protein in the refolding buffer that is considered. This is a significant variable, but we have shown here that the concentration of denatured protein and the solvent conditions prior to refolding are also significant. The first few milliseconds after addition of the denatured protein to the renaturation medium have been suggested as being the most critical [15]. During this time, the protein is still undergoing dispersion and the actual concentration of refolded protein will lie somewhere between a perfectly mixed system and the initial concentration of denatured protein. Therefore choosing the correct initial conditions is just as critical as choosing the correct final conditions. The initial conditions also dictate the final denaturant and reducing agent concentrations both which have been shown to be important factors in batch refolding.

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REFERENCES

[1] Rudolph, R. (1995) Successful protein folding on an industrial scale, pp. 283-298. In: J. L. Cleland and C. S. Craik (eds). *Protein Engineering Principles and Practices*. John Wiley & Sons, New York, USA.

[2] Thatcher, D. and A. Hitchcock (1994) Protein folding in biotechnology, pp. 229-261. In: R. H. Pain (ed.). *Mechanisms of Protein Folding*, IRLPress/Oxford University Press, Oxford, UK.

[3] Kiefhaber, T., R. Rudolph, H. Kohler, and J. Buchner (1991) Protein aggregation *in vitro* and *in vivo*: A quantitative model of the kinetics competition between aggregation and refolding. *Bio/Technology* 9: 825-829.

[4] De-Young, L., K. Dill, and A. Fink (1993) Aggregation and denaturation of apomyoglobin in aqueous urea solutions. *Biochem.* 32: 3877-3886.

[5] Fahey, E. M., J. B. Chaudhuri, and P. Binding (2000) Refolding and purification of a urokinase plasminogen activator fragment by chromatography. *J. Chromatogr. B*737: 225-235.

[6] De Bernardez Clark, E., D. Hevehan, S. Szela, and J. Maachupalli-Reddy (1998) Oxidative renaturation of hen egg white lysozyme. Folding vs aggregation. *Biotechnol. Prog.* 14: 47-54.

[7] Rudolph, R., U. Opitz, F. Hesse, R. Riebland, and S. Fischer (1992) Reactivation of microbially produced human tissue-type plasminogen activator. *Biotechnol. Int.* 321-325.

[8] Cleland, J. and D. Wang (1990) Cosolvent assisted protein refolding. *Bio/Technology* 8: 1274-1278.

[9] London, J., C. Skrzynia, and M. Goldberg (1974) Renaturation of *Escherichia coli* tryptophanase after exposure to 8 M urea. *Eur. J. Biochem.* 47: 409-415.

[10] Builder, S. and J. Ogez (1984) Purification and activity assurance of precipitated heterologous proteins. *US Patent* 4,620,948.

[11] Vicik, S. and E. De Bernardez-Clark (1991) An engineering approach to achieving high-protein refolding yields, pp. 180-196. In: E. De Bernardez-Clark and G. Georgiou (eds.) *ACS Symposium Series, Vol. 470: Protein Refolding*, American Chemical Society, Washington, DC, USA.

[12] Batas, B. and J. B. Chaudhuri (1996) Protein refolding at high concentration using size exclusion chromatography. *Biotechnol. Bioeng.* 50: 16-23.

[13] Werner, M., M. Clore, A. Gronenborn, A. Kondoh, and R. Fisher (1994) Refolding proteins by gel filtration chromatography. *FEBS* 345: 125-130.

[14] Buchner, J. and R. Rudolph (1991) Renaturation, purification and characterisation of recombinant fab-fragments produced in *Escherichia coli*. *Bio/Technology* 9: 157-162.

[15] Goldberg, M., R. Rudolph, and R. Jaenicke (1991) A kinetic study of the competition between renaturation and aggregation during the refolding of denatured-reduced egg white lysozyme. *Biochem.* 30: 2790-2797

[16] Tanford, C., R. Pain, and N. Otchin (1966) Equilibrium and kinetics of the unfolding of lysozyme by guanidine hydrochloride. *J. Mol. Biol.* 15: 489-504

[17] Chaffotte, A., Y. Guillou, and M. Goldberg (1992) Kinetic resolution of the peptide bond and side chain far-uv circular dichroism during the folding of hen egg-white lysozyme. *Biochem.* 25: 6965-6972.

[18] Epstein, C. and R. Goldberger (1963) The oxidative refolding of denatured reduced lysozyme. *J. Biochem.* 238: 1380-1388.

[19] Saxena, V. and D. Wetlaufer (1970) Formation of the three dimensional structure in proteins. Rapid nonenzymatic reactivation of reduced lysozyme. *Biochem.* 9: 5015-5022.

[20] Wetlaufer, D. B., E. R. Johnson, and L. M. Clauss (1974) pp 269-280. In: E. F. Osserman, R. E. Canfield, and S. Beychok (eds.). *Lysozyme*. Academic Press, New York, USA.

[21] Morsky, P. (1983) Turbidimetric determination of lysozyme with *Micrococcus lysodeikticus* cells. Re-examination of the reaction conditions. *Anal. Biochem.* 128: 77-85.

[22] Khechinashvilli, N., P. Privalov, and E. Tiktopulo (1973) Calorimetric investigation of lysozyme thermal denaturation. *FEBS Lett.* 30: 57-60.

[23] Privalov, P. (1992) Physical basis of the stability of the folded conformations of proteins, pp 83-126. In: T. E Creighton (ed). *Protein Folding*. W H Freeman and Co, New York, USA.

[24] Fischer, B., I. Sumner, and P. Goodenough (1993) Renaturation of lysozyme - temperature dependence of renaturation rate, renaturation yield and aggregation: Identification of hydrophobic folding intermediates. *Arch. Biochem. Biophys.* 306: 183-187.

[25] Jaenicke, R. and R. Rudolph (1989) Folding proteins pp. 191-223. In T. E. Creighton (ed.). *Protein Structure - A Practical Approach*. IRL Press, Oxford, UK.

[26] Mendoza, J., E. Rogers, G. Lorimer, and P. Horowitz (1991) Unassisted refolding of urea unfolded rhodanese. *J. Biol Chem.* 266: 13587-13591.

[27] Gatenby, A., P. Viitanen, and G. Lorimer (1990) Chaperonin assisted polypeptide folding and assembly: Implications for the production of functional proteins in bacteria. *Trends Biotechnol.* 8: 345-358.

[28] Buchner, J. and R. Rudolph. (1991) Renaturation, purification and characterisation of recombinant fab-fragments produced in *Escherichia coli*. *Bio/Technology* 9: 157-162.

[29] Seckler, R., A. Fuchs, J. King, and R. Jaenicke. (1989) Reconstitution of the thermostable trimeric phage-P22 tailspike protein from denatured chains *in vitro*. *J. Biol. Chem.* 246: 11750-11753.

[30] Dill, K. (1990) Dominant forces in protein folding. *Biochem.* 29: 7133-7155.

[31] Viitanen, P., T. Lubben, J. Reed, P. Goloubinoff, D. O'Keefe, and G. Lorimer (1990) Chaperonin-facilitated refolding of ribulosebisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (gro EL) are K^+ dependent. *Biochem.* 29: 5665-5671.

[32] Matsubara, M., D. Nohara, and T. Sakai (1992) Difference between guanadinium chloride and urea as denaturants of globular proteins and the possibility of application to improved refolding processes. *Chem. Pharm. Bull.* 40: 550-552.
[33] Denton, M. E., D. M. Rothwarf, and H A Scheraga (1994) Kinetics of folding of guanidine-denatured hen egg-white lysozyme and carboxymethyl(cys(6),cys(127))-lysozyme: A stopped-flow absorbency and fluorescence study. *Biochem.* 33: 11225-11236.
[34] Horowitz, P. and N. Criscimagna (1986) Low concentrations of guanidinium chloride expose apolar surfaces and cause differential perturbation in catalytic intermediates in rhodanese. *J. Biol. Chem.* 261: 15652-15658.
[35] Tsou, C.-L. (1986) Location of the active sites of some enzymes in limited and flexible molecular regions. *Trends Biochem. Sci.* 11: 427-429.
[36] Tsou, C.-L. (1993) Conformational flexibility of enzyme active sites. *Science* 262: 380-381.
[37] Damodaran, S. (1987) Influence of solvent conditions on the refolding of bovine serum albumin. *Biochim. Biophys. Acta* 914: 114-121.
[38] Zettlemeissel, G., R. Rudolph, and R. Jaenicke (1979) Reconstitution of lactic dehydrogenase. Non-covalent aggregation vs. reactivation *Biochem.* 18: 5567-5571.
[39] Shoemaker, J., A. Brasnett, and F. Marston (1985) Examination of calf prochymosin accumulation in *E. coli*. *EMBO J.* 4: 775.
[40] Rudolph, R. (1990) Renaturation of recombinant, disulfide-bonded proteins from 'inclusion bodies', pp.149-171. In: H. Tschesche (ed.). *Modern Methods in Protein and Nucleic Acid Research.* Walter de Gruyter, Berlin, Germany.

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