ELSEVIER

Journal of Chromatography A, 766 (1997) 109–119

JOURNAL OF CHROMATOGRAPHY A

Studies of the hydrodynamic volume changes that occur during refolding of lysozyme using size-exclusion chromatography

Boris Batas, Huwel R. Jones, Julian B. Chaudhuri*

School of Chemical Engineering, University of Bath, Bath BA2 7AY, UK

Received 11 October 1996; accepted 25 November 1996

Abstract

A size-exclusion chromatography-based refolding process (SEPROS) has successfully been used to renature lysozyme at high concentrations. This process is based on the different hydrodynamic characteristics of folded and unfolded proteins and their interaction with gel filtration media. In this paper we have quantified the changes in Stokes radius, hydrodynamic volume and partition coefficient that occur when lysozyme is refolded from urea in a size-exclusion column. In 8 *M* urea partially folded and unfolded lysozyme were resolved using Superdex 75 HR. These two species were present at approximately the same concentration. As the urea concentration was decreased the unfolded species gradually decreased until at 4 *M* urea only partially folded lysozyme remained, which continued to fold on further reduction of the urea concentration. Using these results the initial mechanism for size exclusion chromatography protein refolding has been confirmed.

Keywords: Protein folding; Hydrodynamic volume; Lysozyme; Proteins

1. Introduction

The production of recombinant proteins using bacteria such as *E. coli* is technically straight forward. However, in many cases the protein accumulates at high levels as an insoluble aggregate or inclusion body within the cell [1]. Inclusion bodies have no biological activity. Protein refolding is the series of operations that involves isolating the inclusion bodies, dissolving them in strong denaturants and then recovering the biological activity by the controlled removal of the denaturant [2]. A major obstacle to achieving high refolding yields is the propensity of the solubilised, unfolded proteins to form irreversible aggregates rather than to proceed to the fully folded native state [3]. These aggregates form through interactions between the exposed hydrophobic residues that in the native state are buried in the core of the protein. Physical strategies to enhance protein refolding rely on separating refolding molecules until they are in a stable, nonaggregating conformation (e.g., dilution or fed-batch refolding) or blocking the protein–protein interactions using polyethylene glycol [4] or L-arginine [5].

We have recently reported a new method to improve refolding yields and to increase the concentration of refolded proteins in a single operation [6]. This method utilises size exclusion chromatography matrices to perform controlled buffer exchange from solubilising to refolding solutions, aggregate removal and the folding reaction. The reduced diffusion of proteins in gel-filtration media has been

*Corresponding author.

PII S0021-9673(96)01020-5

shown to suppress the nonspecific interactions of partially folded molecules thus reducing aggregation. Hen egg white lysozyme (HEWL) and bovine carbonic anhydrase (CAB) were successfully refolded from initial protein concentrations of up to 80 mg/ml using Sephacryl S-100 (HR). The aggregation reaction for lysozyme was reduced and was only detected at the highest protein concentration used.

A similar approach to the refolding of secretory leukocyte protease inhibitor (SLPI) has also been reported [7]. In this study refolding was carried out using a rolled stationary phase of DEAE-cellulose as a cylinder in a chromatography column. The ion-exchange nature of this matrix was suppressed by the addition of 500 m*M* NaCl, and thus separation of SLPI from the denaturant and reducing agent occurred by a size-exclusion mechanism.

From our experimental observations of protein refolding in a size-exclusion column we proposed the following mechanism for size-exclusion chromatography-based refolding [6]. Initially, the denatured unfolded protein has a random coil configuration and thus a large hydrodynamic radius; the protein is therefore excluded from the bulk of the bead pores. When the eluent (refolding buffer) is applied the local solvent environment becomes lower in the denaturant, and the protein starts to fold. This results in a smaller hydrodynamic radius as the protein develops a more compact and native-like structure. At this stage the protein can move further into the bead in a partially folded form. It should be noted that the partition coefficient of protein between the mobile phase and the gel increases over time as the protein folds and becomes more compact. Because of the porous nature of the gel media, transport of the protein into the pores is diffusion-limited; this will act to help refolding by reducing intermolecular aggregation. Within the pores the refolding reaction is completed and there is minimal likelihood of protein aggregation. The protein is finally eluted from the column as an active native protein. The size exclusion nature of this process ensures that any aggregates that do form are removed from the column first, and the chaotrope species which have a small molecular weight in comparison to the protein are eluted after the refolded protein.

Under equilibrium conditions which allow unfolding and refolding to occur, it is possible to separate and study the folded and unfolded fractions without significantly disturbing the folding equilibrium using size-exclusion HPLC (SEC-HPLC) [8–10]. Where the time scale for refolding is slower that the chromatographic separation this method allows one to observe the native and denatured states of protein molecules simultaneously [9]. The changes in protein size as measured by SEC-HPLC have been validated using circular dichroism measurements thus enabling the use of this method as a tool for monitoring changes in protein size and conformation [9,10]. In this work, we have tested the mechanism of protein refolding in gel filtration media, using SEC-HPLC to measure the changes in the hydrodynamic radius of lysozyme. We have carried out all of our measurements in refolding buffer with decreasing concentrations of denaturant, that match those found in the size-exclusion-based refolding process.

2. Materials and methods

2.1. Chemicals

Crystalline hen egg white lysozyme (HEWL), bovine serum albumin (BSA), bovine carbonic anhydrase B (BCAB), Blue dextran, *Micrococcus lysodeikticus* dried cells, reduced and oxidised glutathione (GSH, GSSG) and urea were obtained from Sigma Chemicals Co. Ltd. All other chemicals were analytical grade. Water used for the experimental work was ultrapure water obtained from a reverse osmosis water system (Elgastat Prima).

2.2. Refolding lysozyme using size-exclusion chromatography

This method is described fully in Batas and Chaudhuri [6]. Briefly, the system consisted of the packed column, a variable speed peristaltic pump (Watson–Marlow), a fraction collector (Pharmacia, RediFrac), a UV detector (ACS, 750/11AZ) and a chart recorder (Phillips, PM 8251). Lysozyme was denatured by incubating the protein in 0.1 *M* Tris–HCl, pH 8.6 containing 8 *M* urea and 0.15 *M* DTT for 2 h at 20°C. A 1.5 ml sample of denatured lysozyme (40 mg/ml) was applied onto a XK 26/

100 column (Pharmacia Biotech) packed with 530 ml of Sephacryl S-100 and previously equilibrated with refolding buffer. The sample was eluted at a flowrate of 2.5 ml/min at 20°C, using refolding buffer. Fractions were collected and analyzed for enzyme activity.

2.3. Hydrodynamic radius measurements using HPLC

All experiments were carried out with a Superdex 75 HR 10/30 prepacked column (Pharmacia Biotech, St. Albans, UK). Superdex 75 HR has a globular protein separation range of 3000–70 000 kDa; the average particle size is 13 μm. This column was used with a Gilson HPLC system under the control of a Gilson 712 HPLC System and control software. A Rheodyne 7125 injection valve with 1 ml sample loop was used for sample injection. All experiments were conducted at 20°C and all solutions used with the HPLC were degassed and filtered using a 0.2 μm nylon filter. Measurements were made using a Gilson 115 UV detector connected to a data acquisition package (PCL-818L, Advantech). All experiments were carried out in duplicate. The chromatographic peaks were analyzed using a Gaussian fit algorithm (Microcal Origin 3.5, Northampton, MA, USA). The mass of protein in each fraction was determined by dividing the area under each peak by the extinction coefficient of lysozyme at the monitored wavelength.

2.3.1. Column calibration

The total and void volumes of the column were determined using Blue dextran 2000 and a 10 mg/ml acetone solution. The following proteins were used for column calibration under a variety of conditions: hen egg white lysozyme (HEWL); bovine carbonic anhydrase B (BCAB) and bovine serum albumin (BSA). The buffers used for each protein are listed in Table 1. To produce unfolded proteins for column calibration, 10 mg of each protein was incubated in 10 ml of the respective buffer at 4°C for 24 h. The concentration of the protein solution was adjusted to approximately of 1 mg/ml. The protein solutions were further degassed and filtered prior to application on the HPLC column. The column was equilibrated with the respective buffer for 1 h prior to protein-loading. The flow rate of the mobile phase through the column was maintained at 0.5 ml/min.

2.3.2. Measurement of the Stokes radius of lysozyme

Solutions of urea (2–8 *M*) and reduced and oxidised glutathione present at a molar ratio of 10:1, were prepared in 0.1 *M* Tris–HCl, pH 8.6. Ten mg of HEWL was incubated at 4°C for 24 h in 10 ml of buffer. The concentration of the protein solution was diluted to 1 mg/ml. The column was equilibrated with the respective buffer for 1 h prior to protein buffer injection. The mobile phase was identical to the incubating buffer, and its flow rate through the column was maintained at 0.5 ml/min. All experiments were carried out in duplicate, and the maxi-

Table 1
Buffers used for column calibration

Buffer	Composition	Reference
Lysozyme		[10]
Native state	0.1 *M* sodium phosphate, pH 6.8, 4°C	
Unfolded	0.1 *M* sodium phosphate, 8.0 *M* urea, 0.01 *M* DTT, pH 6.8, 4°C	
BCAB		[10]
Native state	0.1 *M* sodium phosphate, pH 6.8, 15°C	
Unfolded	0.1 *M* sodium phosphate, 8.0 *M* urea, pH 6.8, 15°C	
BSA		[9]
Native state	0.2 *M* NaCl, 0.01 *M* Tris, 1.0 m*M* EDTA, pH 7.0	
Unfolded	6.0 *M* GdmCl, 0.01 *M* DTT, 1.0 m*M* EDTA, pH 7.0	

mum error of the average measurements was less than 0.5%.

2.4. Analytical methods

Lysozyme concentration was measured using A_{280} values using an absorbance for 1 mg/ml of 2.63 (cell path length=1 cm) for native lysozyme and 2.37 for reduced, denatured lysozyme [11].

The activity of native and refolded lysozyme (HEWL) was determined at 25°C by following the decrease in absorbance at 450 nm of a 0.25 mg/ml. *Micrococcus lysodeikticus* suspension in 0.06 *M* potassium phosphate, pH 6.2 [12]. The assay volume was 3 ml and between 10 and 50 μl of protein sample was added. One unit of activity corresponds to an absorbance decrease of 0.0026 per minute. The activity of renatured enzymes was expressed in percentage of activity with respect to that of the native enzyme at the same concentration in the presence of the same buffer.

The oxidised form of DTT was quantified spectrophotometrically at 280 nm [13], and urea and GdnHCl concentrations were determined using refractive index [14].

3. Results and discussion

3.1. Refolding lysozyme using size-exclusion chromatography

We refolded lysozyme by loading 1.5 ml of a 40 mg/ml solution of denatured protein onto a Sephacryl S-100 column. The protein was eluted using 2 *M* urea in refolding buffer which has been reported as giving the highest refolding yield for lysozyme [15]. Fig. 1 shows the elution profile of two samples of lysozyme. The trace with a single peak is native lysozyme, loaded and eluted with the refolding buffer. This protein has an elution volume of 450 ml. The second trace shows two peaks: the first, at 350 ml, is fully active, refolded lysozyme, and the second is a mixture of DTT and urea.

This figure shows that denatured lysozyme may be fully refolded by performing buffer exchange and refolding on gel filtration media. This is in agreement with our previous work [6]. It is important to note that the refolded lysozyme, which has an activity identical to that of the native protein, elutes quicker than the native lysozyme control. This reflects the changing size of the folding protein on the column. The native lysozyme is not unfolded in

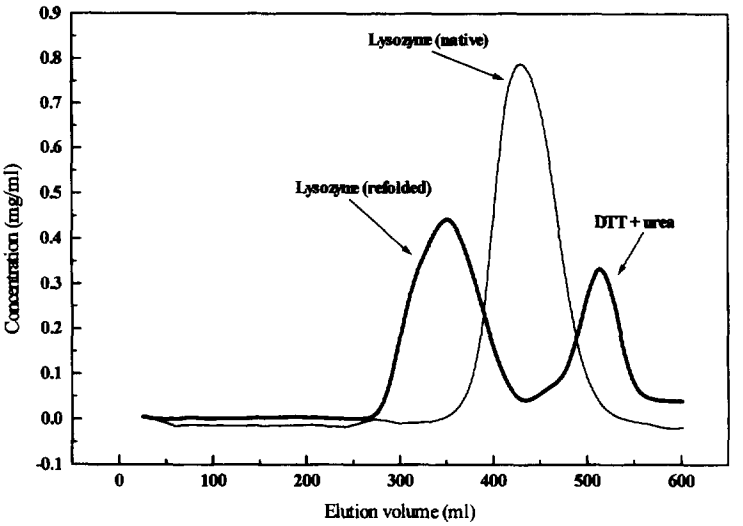

Fig. 1. Lysozyme refolding using size-exclusion chromatography: elution volume of native lysozyme; elution and refolding of denatured lysozyme (bold line).

the mobile phase and so it retains its compact folded conformation and passes through the column in accordance with its partition coefficient. On the other hand the unfolded protein initially has a large hydrodynamic radius and is excluded from the gel media to a greater extent, its local partition coefficient is low at the top of the column. As it moves through the column it refolds as the denaturant gradient changes from 8 to 2 *M* urea. Subsequently the partition coefficient should increase as the molecule forms a more compact native-like structure. The protein becomes more retarded by the column. Thus the elution volume of refolding lysozyme is lower than that of the native molecule under the refolding column conditions. If the refolded lysozyme is recovered and reloaded onto the same column it subsequently elutes at the same elution volume (±0.5%) as native lysozyme (data not shown).

The size-exclusion protein refolding of lysozyme is relatively unaffected by temperature as shown by the data in Table 2. Increasing the column temperature from 20°C to 50°C results in a decrease in the regained specific activity of 5%, and a decrease in the elution volume of 8 ml (2.3%). It should be noted that the majority of the work described herein was carried out at a constant temperature of 20°C, and thus temperature did not significantly affect the reported results.

3.2. Measurement of changes in Stokes radius under refolding conditions using HPLC

3.2.1. Calibration of the Superdex 75HR column for elution volume and Stokes radii

The elution volumes observed with the Superdex column used in this study were calibrated against the known Stokes radii of proteins in the native and denatured states. We calibrated the column with lysozyme, bovine carbonic anhydrase B and bovine serum albumin. The values of the Stokes radii are given in Table 3 [9,10]. The proteins were incubated in the buffers given in Table 1 and were loaded and eluted from the column. The elution profile for each sample was analyzed and from a Gaussian distribution fit, the elution volume of the sample was determined at the centre of the peak. The elution volumes are tabulated in Table 3. A linear relationship was obtained from a plot of the migration rate (1000/elution volume) against the reciprocal of the Stokes radius (Fig. 2):

$$S_r = (-6.3/V_e + 0.94)^{-1} \quad (1)$$

where S_r is the Stokes radius (nm) and V_e is the protein elution volume (ml).

The data in Table 3 is more commonly plotted in the form of the migration rate against the Stokes radius, not its reciprocal as shown here [10,16]. However, in this form our data did not give a linear relationship. It is not known why the reciprocal of both axes produced a linear relationship, however, this may reflect the different methods of measuring the Stokes radius. Nevertheless, Fig. 2 may be used as a calibration plot for this column to estimate the Stokes radius (hydrodynamic radius) of lysozyme or any other macromolecule that does not interact with the column matrix.

3.2.2. Presence of glutathione on lysozyme unfolding in urea

Denaturing solutions of high concentrations of

Table 2
Effect of temperature on size-exclusion refolding of lysozyme

Temperature (°C)	Specific activity (%)	Elution volume (ml)
20	101.1	353
30	93.5	350
40	98.7	346
50	96.2	345

Initial protein concentration = 40 mg/ml.
Native lysozyme specific activity = 99.5% at 25°C.

Table 3
Stokes radii and elution volumes used for column calibration

Protein	Stokes radius[a] (nm)	Elution volume (ml)
Lysozyme native	1.95	15.3
Lysozyme unfolded	2.75	10.4
BCAB native	2.30	12.2
BCAB unfolded	5.05	8.5
BSA native	3.39	9.6
BSA unfolded	8.18	7.8

[a] Data from Refs. [9,10].

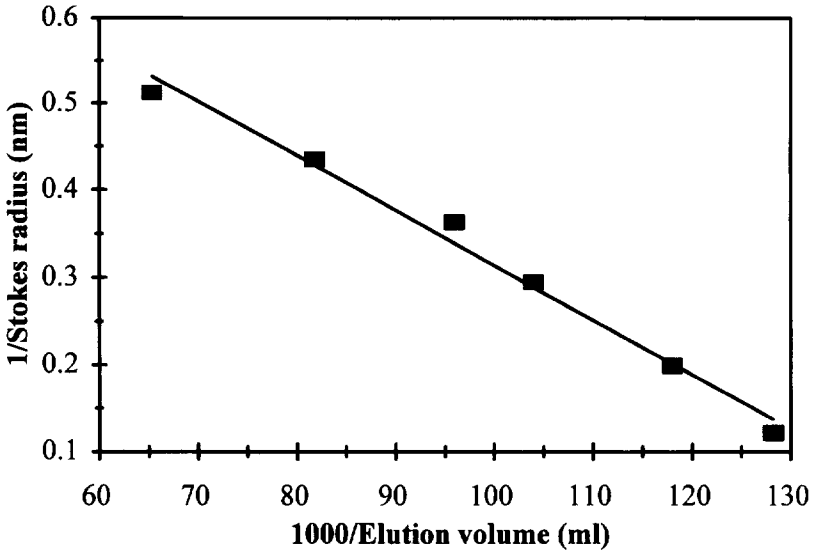

Fig. 2. Correlation of the migration rate (1000/elution volume) of calibration proteins on the reciprocal of Stokes radius.

urea or guanidine hydrochloride have been used to equilibrate and elute proteins where size exclusion chromatography has been used for monitoring refolding processes [8,9,17,18]. In this study, where our aim is to refold the protein, a buffer optimised for refolding lysozyme is used as the mobile phase. We therefore compared the conformational and elution behaviour of lysozyme incubated and eluted in a 8 *M* urea solution in the absence and presence of a redox couple of glutathione. Lysozyme (1 mg/ml) was incubated in two buffers: (1) in 0.1 *M* sodium phosphate, 8.0 *M* urea, 0.15 *M* DTT, in a pH of 6.8, for 24 h at 4°C, and (2) in 0.1 *M* Tris–HCl, 8.0 *M* urea, 3 m*M* reduced and 0.3 m*M* oxidised glutathione, pH of 8.6, for 24 h at 4°C.

From the elution profiles of the denatured lysozyme (Fig. 3) a single peak was observed, whereas in the presence of glutathione (Fig. 4) two peaks were observed: peak 1 at the lower elution volume corresponds to unfolded lysozyme and peak 2 to the folded molecule. Comparison of the Gaussian fit data shows that unfolded lysozyme in Fig. 3 has a slightly lower elution volume of 9.3 ml ±0.08% as compared to a value of 9.5 ml ±0.34% for unfolded lysozyme in the presence of glutathione. In Fig. 4 8 *M* urea was insufficient to fully unfold lysozyme due to the presence of glutathione. Two peaks were eluted, one corresponding to folded and one to unfolded lysozyme. In Fig. 3, DTT completely breaks the disulphide bonds in the protein molecule, whereas the glutathione couple (Fig. 4) was used to catalyse formation of disulphide bonds. Glutathione increases both the rate and the yield of protein reoxidation and thus renaturation by enabling the rapid reshuffling of disulphide bonds at an optimal redox potential. In addition it restricts lysozyme from fully unfolding at a concentration of urea (8 *M*) which fully unfolds lysozyme. From Fig. 4 this suggests that two forms of lysozyme were present in equilibrium in the sample: one form containing disulphide bonds intact, and the second form with broken or mixed disulphide bonds.

The peak corresponding to folded lysozyme has a centre elution volume of 12.4 ml which is lower than the value for native lysozyme (15.3 ml). In the presence of urea the folded molecule is swollen, has a greater hydrodynamic radius and thus elutes earlier. This phenomenon has been observed by Corbett and Roche with myoglobin [9]. This form of the protein should be strictly considered as a partially folded molecule.

3.3. Measurement of Stokes radius as a function of decreasing urea concentration

Gel filtration techniques offer an alternative ap-

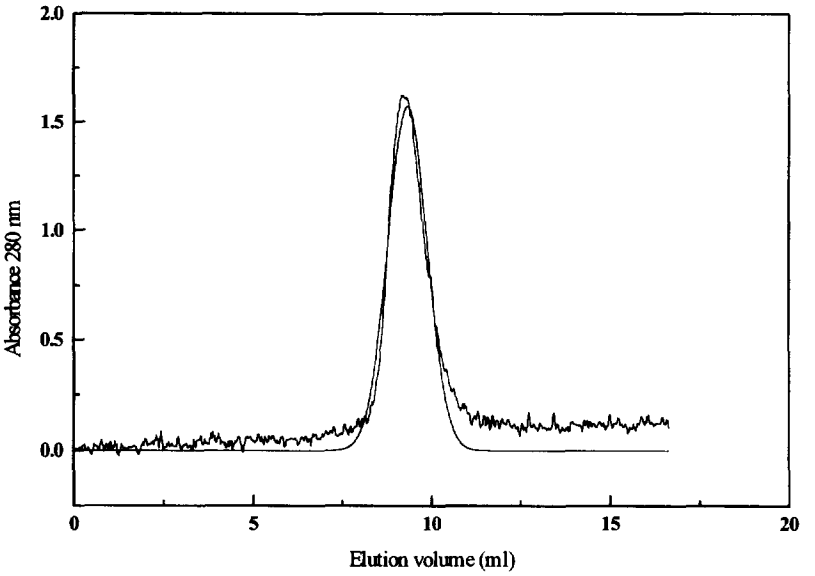

Fig. 3. Elution of lysozyme in 0.1 *M* Tris–HCl, 8.0 *M* urea, 0.15 *M* DTT, pH of 6.8, 4°C, 24 h incubation. The peak corresponds to the unfolded lysozyme. The area under the peak and elution volume are 2.16 and 9.33 ml, respectively.

proach to monitor folding–unfolding equilibria, including the large volume changes associated with these processes. Stable intermediates may be quantified if they are kinetically stable to occur on the same time scale as the chromatography experiment. Analytical size-exclusion chromatography has been used for studying protein unfolding [17,19], however, there are no reports for monitoring refolding

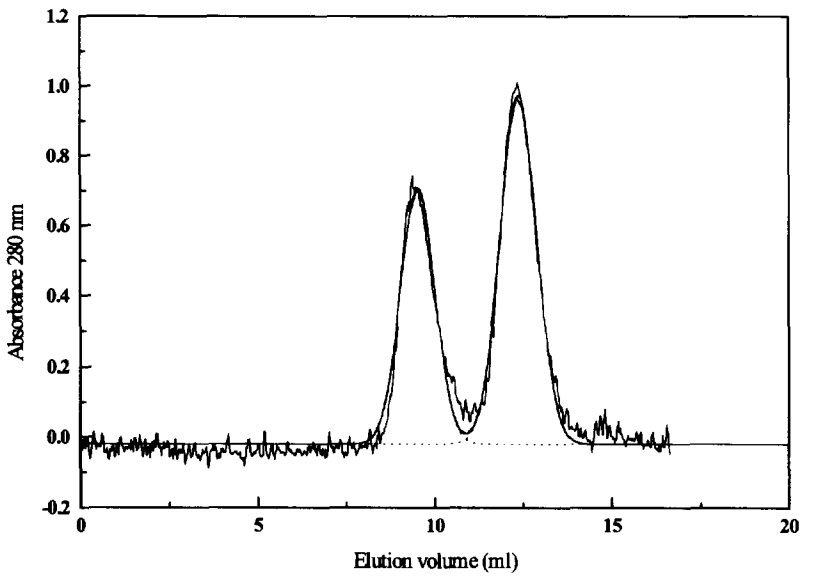

Fig. 4. Elution of lysozyme in 0.1 *M* sodium phosphate, 8.0 *M* urea, 3 m*M* reduced and 0.3 m*M* oxidised glutathione, pH of 8.6, 4°C, 24 h incubation. Peaks 1 and 2 correspond to the partially folded and folded state, respectively. The area under each peak and elution volumes for peak 1 and 2 are 1.08, 9.56 ml, and 1.22 and 12.34 ml, respectively.

under decreasing denaturant concentrations and optimal refolding conditions. We carried out equilibrium studies in a size-exclusion column to investigate how the size of the lysozyme molecule changed in decreasing urea concentrations in the presence of a reduced–oxidised glutathione couple.

Lysozyme was incubated in decreasing concentrations of urea (8–2 *M*), in reduced and oxidised glutathione (3 m*M* reduced: 0.3 m*M* oxidised) and 0.1 *M* Tris–HCl, pH 8.6, for 24 h and then applied onto the column. Based on our previous measurements of unfolding and refolding kinetics, this period of incubation is more than adequate to allow lysozyme to reach equilibrium [6]. A mobile phase identical to the incubation buffer was used for elution. The resulting elution profiles of lysozyme are shown in Fig. 5A–F.

At the highest urea concentration of 8 *M* (Fig. 5A), two symmetrical peaks were observed, with similar areas. The first peak corresponded to the unfolded state of lysozyme and the second to the partially folded state. As the urea concentration decreased, the unfolded protein peak got smaller and by 4 *M* urea only one peak was observed at this and lower concentrations (E–F). As the concentration of urea decreased, the elution volume for the folded conformer increased from 12.4 to 14.9 ml (<0.5% error based on the average of duplicate experiments).

The presence of two peaks at high concentrations of urea indicates that the two states of protein exist in equilibrium, and that the lysozyme denatures on the simple N⇔U scheme [8,9]. Our results show the exchange from the unfolded to the folded forms of lysozyme occurs as the environment favours refolding. This bimodal distribution of the conformers is typical of the "all or none" characteristic unfolding of small globular proteins [10]. The observation of the exchange between the conformers is possible as this is slower than the characteristic time of the chromatographic operation. The increase in elution volume of the partially folded molecule approaches that for native lysozyme (15.3 ml) as the Stokes radius of the molecule decreases from 2.3 to 1.8 nm (Table 4).

The fraction of either conformer population that is denatured, f_d, may be determined using the observed Stokes radius, from the equation

$$f_d = (R_{s,obs} - R_{s,N})/(R_{s,D} - R_{s,N}) \quad (2)$$

where $R_{s,obs}$ is the observed Stokes radius, and $R_{s,N}$ and $R_{s,D}$ are the Stokes radii for the native and denatured states of the protein, respectively. It was assumed that the native and denatured state occurred in 1 *M* urea–glutathione buffer and 8 *M* urea–DTT buffer respectively. This data is plotted for both the folded and unfolded species as a function of urea concentration in Fig. 6. The labels on Fig. 6 refer to the relative percentage of the two species that are present at a particular urea concentration as calculated from the areas under peaks 1 and 2 in Fig. 5.

Fig. 6 shows that for lysozyme in 8 *M* urea, the highest concentration used, the conformers are present in approximately equal amounts. The unfolded protein is approximately 90% denatured (f_d=0.9), and the folded protein is about 20% denatured. As the urea concentration is reduced two changes are apparent: The fraction of the partially folded and unfolded molecules that is denatured reduces; and the relative proportions of the two species decrease until at 4 *M* urea only the partially folded lysozyme is present. At this concentration about 10% of the partially folded lysozyme is denatured, however, as the urea concentration further decreases and f_d reaches zero, no denaturation is present.

The values for the average partition coefficient (K_{av}) and molecule volume were calculated for each state of protein in varying urea concentrations and are tabulated in Table 4. The average partition coefficients are significantly increased for both the folded and unfolded states as the concentration of urea is reduced. As lysozyme folds at each concentration of urea, the molecules contract to a smaller hydrodynamic volume. The volumes of the unfolded molecule in 8 *M* urea, and folded molecule in 1 *M* urea were calculated to be 67.8 and 8.6 nm^3 respectively. The difference in volume between the native protein in 1 *M* urea and the swollen but still globular, folded molecule in 8.0 *M* urea corresponds to a 89% swelling of the folded molecule. The swelling of the unfolded molecule in the range of 4 to 8 *M* urea is 98% which reflects the higher accessibility of the unfolded polypeptide coil domain to the denaturing solvent.

The term "swelling" was used by Corbett and Roche [9] to describe an increase in volume of either the unfolded or folded state of a protein. It was used to support evidence of protein denaturation moni-

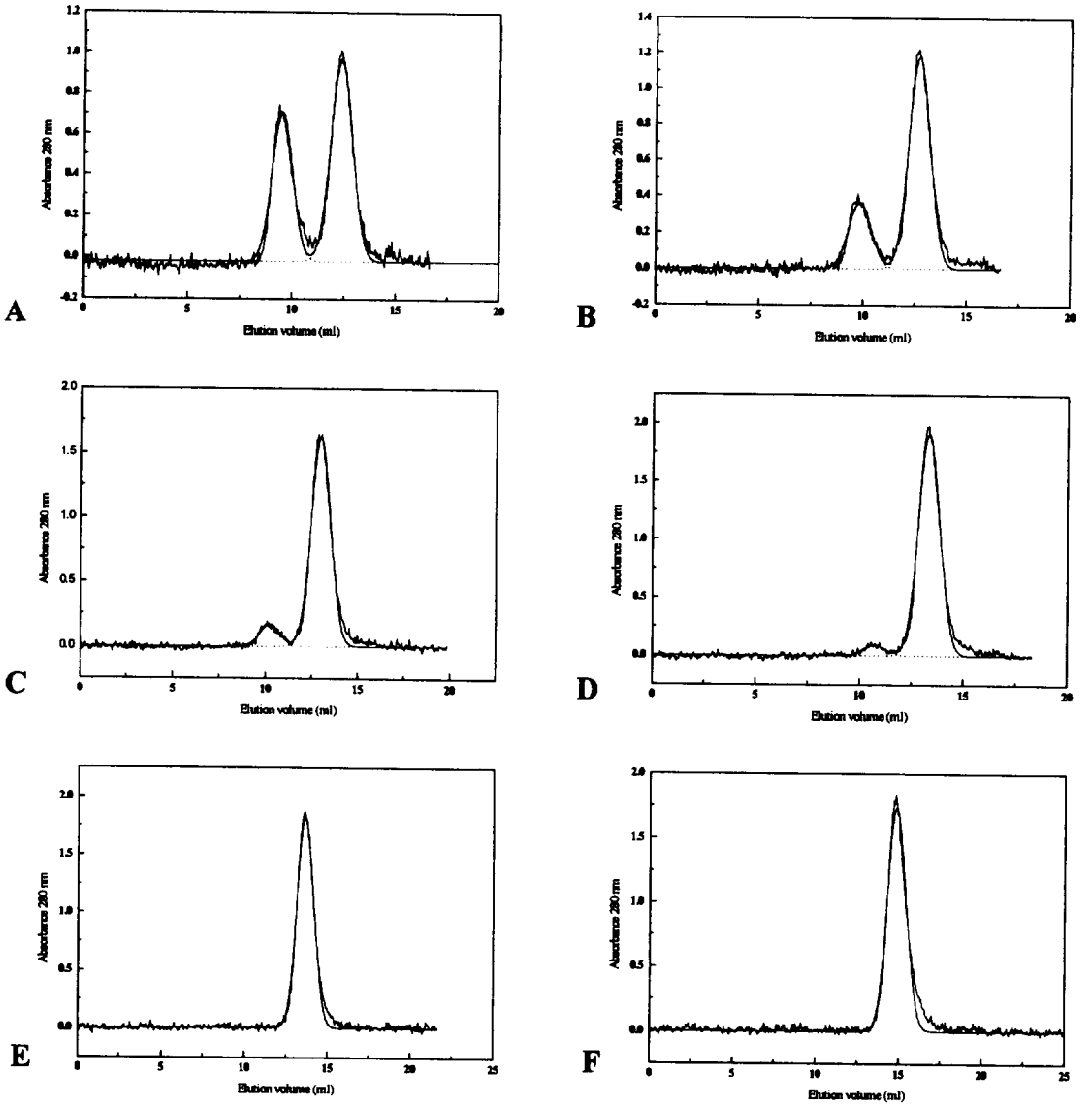

Fig. 5. Lysozyme elution in 0.1 *M* Tris–HCl, 8 *M* urea (A); 7 *M* (B); 6 *M* (C) 5 *M* urea (D); 4 *M* (E); and 2 *M* (F), reduced and oxidised glutathione in a ratio of 10:1, pH 8.6 at 4°C, 24 h incubation. Peak (1) corresponds to the protein molecule in the unfolded state, the second peak represents the partially folded fraction.

tored by increases in the Stokes radius. These workers have shown that the large increases in Stokes radius occur upon unfolding, and that gel filtration offers an alternative method to follow such changes. This is based on a correlation between the protein retention time, measured by size exclusion chromatography, and the Stokes radius of protein molecule [16,20].

The observations of changes in the hydrodynamic radius and volume with urea concentration help to explain the process of refolding using size-exclusion chromatography. When two states of lysozyme are present in equilibrium, the unfolded state has a lower elution volume and a higher Stokes radius to that in the folded state (Table 4 and Fig. 6). This suggests that the physical conformation of lysozyme is re-

Table 4
Change in lysozyme parameters as a function of urea concentration

Lysozyme and urea buffer	Elution volume V_e (ml)		Stokes radius R_s (nm)		Percentage unfolded (%)		Volume of molecule (nm^3)		K_{av}	
	Unfolded	Folded	Unfolded	Folded	Unfolded	Folded	Unfolded	Folded	Unfolded	Folded
8 *M**	9.33		3.70		100.00		67.76		0.10	
8 *M*	9.54	12.39	3.52	2.29	44.21	55.79	58.30	16.03	0.11	0.34
7 *M*	9.84	12.70	3.28	2.23	26.65	74.35	47.14	14.87	0.14	0.37
6 *M*	10.20	12.93	3.05	2.19	11.30	98.70	37.98	13.97	0.17	0.38
5 *M*	10.72	13.03	2.81	2.13	5.24	94.76	29.49	12.81	0.21	0.39
4 *M*		14.08		2.02		100.00		10.97		0.48
3 *M*		14.07		2.02		100.00		10.97		0.48
2 *M*		14.88		1.92		100.00		9.42		0.54
1 *M*		15.52		1.86		100.00		8.57		0.59

* Lysozyme in 8 *M* urea, 0.1 *M* Tris–HCl and 0.15 *M* DTT.
R_s values were calculated from the linear line equation of the calibration plot (Fig. 2).
K_{av} was obtained from the following expression: $K_{av}=(V_e-V_0)/(V_t-V_0)$, where V_e is the elution volume observed, V_0 is the void of the column and V_t is the total volume of the column.
The volume of molecules (V) were calculated from Stokes radius values.

sponsible for the separation of the unfolded and folded state. This agrees with our previous hypothesis that the partially folded protein penetrates the stationary phase to a greater extent in comparison to the unfolded lysozyme thus minimising protein–protein aggregation and increasing the refolding yield. The protein species are further kept apart during elution as the folded state requires a greater volume to elute than does the unfolded protein. The success of this process will depend on the degree of resolution inherent in the size-exclusion media.

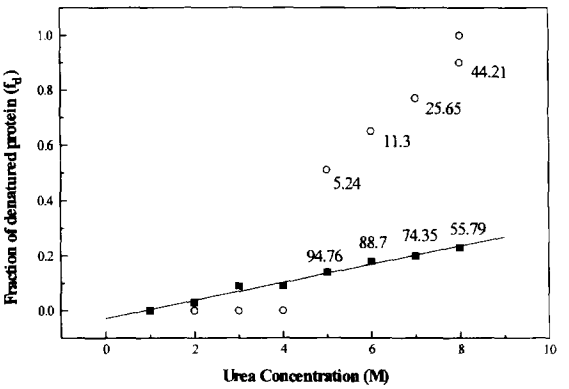

Fig. 6. Relationship of fraction of lysozyme unfolding with urea concentration: unfolded state (○); and folded state (■). The labels represent the percentage of either the unfolded or folded conformers.

Acknowledgments

We gratefully acknowledge the financial support of the Biotechnology and Biological Sciences Research Council through grant GR/J/19801.

References

[1] J.F. Kane and D.L. Hartley, Trends Biotechnol., 6 (1988) 95–101.
[2] D.R. Thatcher, P. Wilks and J.B. Chaudhuri, in N.C. Price (Editor), Proteins Labfax, BIOS Scientific Publishers/Academic Press, Oxford, 1996, pp. 119–130.
[3] G. Georgiou and G. Bowden, in A. Prokop, R.K. Bajpai and C.S. Ho (Editors), Recombinant DNA Technology and Applications, McGraw-Hill, New York, NY, 1991, pp. 333–356.
[4] J.L. Cleland and D.I.C. Wang, Bio/Technology, 8 (1990) 1274–1278.
[5] J. Buchner and R. Rudolph, Bio/Technology, 9 (1991) 157–162.
[6] B. Batas and J.B. Chaudhuri, Biotechnol. Bioeng., 50 (1996) 16–23.
[7] K.H. Hamaker, J. Liu, R.J. Seely, C.M. Ladisch and M.R. Ladisch, Biotechnol. Progr., 12 (1996) 184–189.
[8] A.M. Al-Obeidi and A. Light, J. Biol. Chem., 263 (1988) 8642–8645.
[9] R.J.T. Corbett and R.S. Roche, Biochemistry, 23 (1984) 1888–1894.
[10] V.N. Uversky, Biochemistry, 32 (1993) 13288–13298.
[11] V.P.a.W. Saxena, D.B, Biochemistry, 9 (1970) 5015–5023.

[12] M.E. Goldberg, R. Rudolph and R. Jaenicke, Biochemistry, 30 (1991) 2790–2797.
[13] W.W. Cleland, Biochemistry, 3 (1964) 480–482.
[14] C.N. Pace, Methods Enzymol., 131 (1986) 266–280.
[15] M. Matsubara, D. Nohara, E. Kurimoto, Y. Kuroda and T. Sakai, Chem. Pharm. Bull., 41 (1993) 1207–1210.
[16] L.C. Davis, J. Chromatogr. Sci., 21 (1983) 214–217.
[17] W. Shalongo, R. Ledger, M.V. Jagannadham and E. Stellwagen, Biochemistry, 26 (1987) 3135–3141.
[18] M.H. Werner, G.M. Clore, A.M. Gronenborn, A. Kondoh and R.J. Fisher, FEBS Lett., 345 (1994) 125–130.
[19] W. Shalongo, M.V. Jagannadham, C. Flynn and E. Stellwagen, Biochemistry, 28 (1989) 4820–4825.
[20] G.K. Ackers, J. Biol. Chem., 242 (1967) 3237–3238.