Abstract: Disulfide bond shuffling in the presence of the reducing agents dithiothreitol (DTT) or β-mercaptoethanol (BME) strongly affects the surface properties of lysozyme solutions. The addition of 0.32 mM DTT substantially alters the kinetic dependencies of the dynamic surface elasticity and surface tension relative to those of pure protein solutions. The significant increase in the dynamic surface elasticity likely relates to the cross-linking between lysozyme molecules and the formation of a dense layer of protein globules stabilized by intermolecular disulfide bonds at the liquid/gas interface. This effect differs from the previously described influence of chaotropic denaturants, such as guanidine hydrochloride (GuHCl) and urea, on the surface properties of lysozyme solutions. If both chaotropic and reducing agents are added to protein solutions simultaneously, their effects become superimposed. In the case of mixed lysozyme/GuHCl/DTT solutions, the dynamic surface elasticity near equilibrium decreases as the GuHCl concentration increases because of the gradual loosening of the cross-linked layer of protein globules but remains much higher than that of lysozyme/GuHCl solutions.

Key words: lysozyme, dithiothreitol, guanidine hydrochloride, protein unfolding, dilational surface rheology

1 INTRODUCTION

Disordered or partly disordered protein structures are widespread in various biological and industrial systems\(^ {1-4}\). However, these structures have only recently become the subject of intensive study, and the obtained information remains quite limited.

Several factors lead to the destruction of protein tertiary structure, such as high pressure and temperature, low pH, and high concentrations of special denaturing substances\(^ {5,6}\). The use of different denaturing agents can result in different protein-denaturation mechanisms.

Substances able to destroy protein secondary and tertiary structures can be divided into two main groups. The first includes chaotropic denaturants, such as guanidine hydrochloride (GuHCl) and urea. These substances mainly affect hydrogen bonds at the surface of protein globules and hydrophobic interactions between different amino acid residues but do not affect the molecule’s disulfide bonds\(^ {5}\). Most studies investigating the interactions between proteins and chemical denaturants have focused on systems containing GuHCl or urea\(^ {5,6,7-12}\). The second group comprises reducing agents, such as dithiothreitol (DTT, Fig. 1) and β-mercaptoethanol (BME). These substances attack the disulfide bonds of protein molecules and cause them to shuffle (become disrupted and then undergo random recombination)\(^ {13}\).

The surface properties of protein solutions have been investigated less frequently than their bulk properties because of the limited number of suitable experimental techniques available. Although the interactions between proteins and denaturants in the surface layer are known to strongly influence the surface properties\(^ {11-14}\), the details of these interactions remain unknown.

Recently, the dilational surface rheology was shown to provide additional information on the protein conformations at the liquid/gas interface\(^ {15-18}\). This approach is based on the strong difference between the kinetic dependencies of the dynamic surface elasticity of solutions of globular protein and those of solutions of non-globular or
unfolded proteins. In the former case, the kinetic dependencies are monotonic and resemble the corresponding results for aqueous dispersions of charged solid nanoparticles. In the latter case, the kinetic dependencies are similar to those for solutions of amphiphilic polymers in which the dynamic surface elasticity goes through a local maximum and approaches relatively low values near equilibrium, in agreement with the theory of the surface viscoelasticity of polymer solutions.

In this work, the surface rheological properties of mixed solutions of lysozyme, DTT, and GuHCl were studied for the first time. Although lysozyme is one of the most frequently studied model proteins, information on the structure of its adsorption layers at the liquid/gas interface remains rather controversial. Lysozyme forms small rigid globules in aqueous solutions and belongs to the group of "hard" proteins. It has a molecular weight of 14,300 Da and consists of 129 amino acid residues. Closed-packed lysozyme globules are stabilized by four disulfide bonds, which have dimensions of 4.5 × 3.0 × 3.0 nm, and consist of two main domains. Lysozyme structure is relatively stable against high temperature and chaotropic chemical denaturants, such as urea and GuHCl, probably because of the disulfide bonds connecting the remote amino acid residues. The presence of these bonds between the 6th and 127th residues and between the 30th and 115th residues makes the globule more rigid. The addition of a reducing agent significantly changes the lysozyme's globular structure. Chang et al. described the formation of lysozyme isomers after the addition of DTT or BME and detected many more unfolded globules in the solutions containing both chaotropic and reducing agents than in those containing only a chaotropic denaturant.

The main aim of this work was to determine the influence of lysozyme-denaturant interactions in the surface layer and disulfide bond shuffling on the dilational surface rheological properties and obtain additional information on protein conformations at the liquid/gas interface. Another aim of this work was to compare the surface rheological properties of lysozyme solutions containing a reducing agent with those of lysozyme solutions containing a strong chaotropic denaturant (GuHCl) studied previously.

2 EXPERIMENTAL PROCEDURES

Lysozyme (Sigma-Aldrich Chemie GmbH, Riedstrasse 2 D-89555 Steinheim 49 7329 970, Germany) was used as received. Lysozyme solutions of required concentrations in phosphate buffer at pH 7 were prepared by diluting a 0.05-mM solution. DTT and GuHCl (Sigma-Aldrich Chemie GmbH, Riedstrasse 2 D-89555 Steinheim 49 7329 970, Germany) were used as received. These substances were dissolved in a small quantity of phosphate buffer before being added to the protein solutions. The volume of the solution was then increased to the required value.

The pH of all solutions was adjusted to 7 by adding components of the Na2HPO4–NaH2PO4 buffer system (Sigma-Aldrich Chemie GmbH, Riedstrasse 2 D-89555 Steinhelm 49 7329 970, Germany). The ionic strength of all solutions was 0.04 M. The solutions were prepared using triply distilled water. A glass apparatus was used in the last two steps of the distillation. The surface tension of the pure buffer solution was 72.8 mN/m.

All lysozyme solutions were used without storage, and measurements of the surface properties were started within several minutes after preparing a fresh solution. All measurements were performed at 20 ± 1°C.

The dynamic dilatational surface elasticity was measured using the oscillating ring method. The surface of the solution under investigation was periodically expanded and compressed by the oscillations of a glass ring along its axis. The ring was partly immersed into the liquid with its axis perpendicular to the liquid surface, and its internal surface was grounded to improve wetting. The ring oscillations led to regular oscillations of the liquid surface area and the surface tension of the solution because of periodical changes of the meniscus shape at the internal surface of the ring. The surface tension of the investigated liquid was measured inside the ring using the Wilhelmy plate method. The main advantage of the oscillating ring technique is that it creates almost pure dilational deformations of the liquid surface and therefore contributes negligible shear stresses to the experimental results. The relative amplitude and frequency of the solution surface area oscillations were 10% and 0.1 Hz, respectively.

The real ε ′ and imaginary ε ″ components of the dilational dynamic surface elasticity ε were calculated from the amplitudes of the oscillations of the surface tension δγ and surface area δS and the phase shift φ between the oscillations of these two quantities by the following relation:

\[ \varepsilon = \frac{dy}{d \ln S} = \varepsilon_1 + i \varepsilon_2 = \frac{-S \delta \gamma}{\delta S} \cos \phi + i \frac{S \delta \gamma}{\delta S} \sin \phi \]  

The imaginary part of the complex dynamic surface elasticity of the investigated solutions was much smaller than the real part. Therefore, only the results for the real part are discussed below. The experimental errors of the oscillating ring method are mainly determined by the errors in the surface tension measurements and are less than ±5%. Furthermore, the surface dilational rheology measurements were repeated a few times to minimize the random error.

3 RESULTS AND DISCUSSION

The dynamic surface properties of lysozyme/DTT and lysozyme/GuHCl/DTT solutions were measured as a function
of surface age and chaotropic denaturant concentration at constant lysozyme and DTT concentrations of 0.005 mM and 0.32 mM, respectively, at pH 7. The surface properties at this low-protein concentration change over a few hours, and the different steps of the adsorption layer formation could be observed. The adsorption of positively charged lysozyme globules at pH 7 was slower than that of neutral amphiphilic polymers at similar concentrations, likely because of an electrostatic adsorption barrier. The adsorption of the first protein molecules generates additional charge at the interface, increasing the repulsion experienced by other adsorbing molecules with similar charges.

The addition of DTT to lysozyme solutions dramatically changed the kinetic dependencies of the dynamic surface elasticity (Fig. 2) and surface tension (Fig. 3). The strong increase in the surface activity of lysozyme and accelerating decrease of the surface tension under the influence of DTT (Fig. 2) could be attributed to the loosening of the protein tertiary structure and an increased number of mobile hydrophobic groups able to move to the boundary with the gaseous phase during adsorption. A similar strong decrease in the equilibrium surface tension of lysozyme solutions was recently observed in the presence of urea. In both cases, the surface tension decreased to 46–47 mN/m. Notably, this loosening of the protein tertiary structure under the influence of urea does not produce completely unfolded globules but rather a special molten globule state. The altered lysozyme structure in lysozyme/DTT solutions could lead to more rapid decreases in surface tension because of several effects. First, the protein molecules occupied larger areas of the liquid-gas interface, and thus, the surface tension started to decrease at lower surface concentrations than when compact globules adsorb, leading to the disappearance of the induction period (Fig. 3). Second, the lower surface concentration decreased the surface charge density, the repulsive forces at the interface, and the adsorption barrier. Furthermore, the addition of DTT could directly influence the lysozyme molecule adsorption rate. Note that similar behavior was observed when NaCl was added.

The surface elasticity near equilibrium increased by more than twofold, from approximately 70 mN/m for pure protein solutions to more than 145 mN/m for lysozyme/DTT solutions (Fig. 2). The growth of the dynamic surface elasticity was also strongly accelerated under the influence of DTT. Note that urea and GuHCl decrease the dynamic surface elasticity of lysozyme solutions. Additionally, no kinetic dependencies of the dynamic surface elasticity had local maxima, and they remained monotonic, unlike the corresponding results for lysozyme/GuHCl solutions, where the local maxima were related to globule unfolding. Thus, the lysozyme globular structure was probably mostly preserved in the surface layer.

The obtained results indicate the formation of a rigid structure in the surface layer, which is compatible with relatively high hydrophobic group mobility inside globules and thus with the loosening of the structure of individual globules. This behavior may be explained by the aggregation and cross-linking of the adsorbed lysozyme molecules under the influence of DTT with partial preservation of their globular structure and thus the formation of a relatively dense elastic surface layer of protein globules stabil...
lized by a net of intermolecular disulfide bonds. This is possible because of the specific conditions at the liquid/gas interface, particularly the higher surface concentration and shorter distances between neighboring lysozyme molecules relative to the bulk phase. In the bulk, at low concentrations, the globules are mostly unable to form aggregates through intermolecular disulfide bonds, and only isomers can be detected\(^{26}\). Therefore, the presence of reducing agent in protein solutions exerts the opposite effect of chaotropic denaturants \((\text{GuHCl and urea})\). In the latter case, the dynamic surface elasticity value near equilibrium decreases steadily as the denaturant concentration increases\(^{27}\).

When both DTT and GuHCl were added to lysozyme solutions simultaneously, the superposition of the two opposite effects could be observed. Even at fairly low concentrations \((0.5 \text{ M})\), the chaotropic denaturant (GuHCl) leads to a strong decrease in the dynamic surface elasticity near equilibrium, followed by a gradual decrease in the elasticity as the denaturant concentration increases (Fig. 4). These changes indicate significant loosening of the adsorption layer structure under the influence of the chaotropic agent compared to that in lysozyme/DTT solutions. As the GuHCl concentration increased from 4 M to 6 M, a local maximum of the kinetic dependence of the dynamic surface elasticity appeared. This effect was typically observed during the unfolding of protein globules when the unfolded protein chains penetrated the subphase and formed loops and tails there. Additionally, the dynamic surface elasticity near equilibrium at different concentrations of the chaotropic denaturant was much higher than that at the same GuHCl concentrations in lysozyme/GuHCl solutions. This behavior indicated that a relatively dense layer containing intermolecular disulfide bonds between protein molecules was also formed in lysozyme/GuHCl/DTT solutions and that some bonds were preserved, even at high GuHCl concentrations. Furthermore, the adsorption layer underwent substantial loosening and even partial unfolding at GuHCl concentrations exceeding 4 M.

The equilibrium surface tension increased as the GuHCl concentration increased and reached typical values for solutions without DTT \((\sim 57 \text{ mN/m at 6 M GuHCl, Fig. 5})\). GuHCl is known to influence the surface activity of lysozyme only slightly\(^{27}\), unlike other globular proteins\(^{15-17}\), but in lysozyme/DTT solutions, it suppresses the surface activity. This peculiarity can probably be attributed to the decreased concentration of non-aggregated hydrophobic groups in the proximal region in the surface layer rather than to the decreased total adsorbed amount of lysozyme. The increasing hydrophobic interactions between some amino acid residues of the lysozyme molecule when its globular structure was disturbed by interactions with GuHCl can decrease the number of hydrophobic groups in contact with the gas phase. Additionally, the formation of loops and tails could lead to the desorption of some hydrophobic groups.

Another peculiarity of the dynamic surface properties of lysozyme/GuHCl/DTT solutions was that the rate of change of the surface properties was strongly increased when the GuHCl concentration increased from 2 M to 4 M (Figs. 4 and 5). The corresponding increase in the solution ionic strength was insufficient to explain this effect. Note that the destruction of lysozyme tertiary structure occurs in this exact concentration range\(^{27}\). These structural changes resulted in a new adsorption mechanism and increased the adsorption rate.

![Fig. 4](image1.png)  
Fig. 4  Kinetic dependencies of the dynamic surface elasticity of lysozyme/GuHCl/DTT solutions at GuHCl concentrations of 0 (violet stars), 0.5 (black squares), 2 (red circles), 4 (green diamonds), 6 M (blue hexagons).

![Fig. 5](image2.png)  
Fig. 5  Kinetic dependencies of the dynamic surface tension of lysozyme/GuHCl/DTT solutions at GuHCl concentrations of 0 (violet stars), 0.5 (black squares), 2 (red circles), 4 (green diamonds), 6 M (blue hexagons).
4 CONCLUSIONS

In summary, measurements of the kinetic dependencies of the dynamic surface properties of mixed lysozyme/DTT and lysozyme/GuHCl/DTT solutions show that a strong reducing agent (DTT) induces aggregation and cross-linking of protein molecules at the solution/air interface and the formation of a dense elastic adsorption layer of lysozyme globules stabilized by a net of intermolecular disulfide bonds. The influence of DTT on the surface properties of lysozyme solutions manifests in a dramatic increase in the protein surface activity and the dynamic surface elasticity of solutions, contrary to the influence exerted by a chaotropic denaturant (GuHCl). The simultaneous addition of DTT and GuHCl leads to the formation of a cross-linked layer of relatively flexible, partially unfolded globules.

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