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# A Kinetic Study of the Competition between Renaturation and Aggregation during the Refolding of Denatured-Reduced Egg White Lysozyme†

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ABSTRACT: The recovery of proteins following denaturation is optimal at low protein concentrations. The decrease in yield at high concentrations has been explained by the kinetic competition of folding and "wrong aggregation". In the present study, the renaturation-reoxidation of hen and turkey egg white lysozyme was used as a model system to analyze the committed step in aggregate formation. The yield of renatured protein for both enzymes decreased with increasing concentration in the folding process. In addition, the yield decreased with increasing concentrations of the enzyme in the denatured state (i.e., prior to its dilution in the renaturation buffer). The kinetics of renaturation of turkey lysozyme were shown to be very similar to those of hen lysozyme, with a half-time of about 4.5 min at 20 °C. The rate of formation of molecular species that lead to formation of aggregates (and therefore fail to renature) was shown to be rapid. Most of the reaction occurred in less than 5 s after the transfer to renaturation buffer, and after 1 min, the reaction was essentially completed. Yet, by observing the effects of the delayed addition of denatured *hen* lysozyme to refolding *turkey* lysozyme, it was shown that folding intermediates become resistant to aggregation only much more slowly, with kinetics indistinguishable from those observed for the appearance of native molecules. The interactions leading to the formation of aggregates were nonspecific and do not involve disulfide bonds. These observations are discussed in terms of possible kinetic and structural aspects of the folding pathway.

Refolding denatured polypeptide chains in vitro has been an important issue for both basic research and applied biotechnology. Indeed, for several decades, the major advances toward the understanding of protein folding [the "second translation of the genetic message" (Goldberg, 1985)] have come from studies of the renaturation of proteins denatured by urea, guanidine, pH, or heat. Recently, with the advent of recombinant DNA technology, it has become simple to express a gene of interest in a foreign cell and to obtain large amounts of the desired polypeptide chain. However, such polypeptide chains are often produced as insoluble, biologically inactive "inclusion bodies" (Marston, 1986; Jaenicke, 1987). To obtain the functional protein from the inclusion bodies, it is usually necessary to dissolve the inclusion bodies in a denaturing medium and to submit the solubilized protein to a renaturation procedure (Kane & Hartley, 1988; Rudolph, 1990). Ideally, renaturation procedures in basic research and

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in industrial application should be performed at high protein concentration. In basic research, high protein concentrations would facilitate the detection of folding intermediates; in industry, reducing the volumes of solutions to be handled would reduce the production time and cost. However, it is a common observation that the yield of renatured protein decreases, sometimes drastically, when one increases the concentration of the protein to be renatured (Teipel & Koshland, 1971; Jaenicke, 1974; London et al., 1974; Orsini et al., 1975; Zettlmeissl et al., 1979). Thus, when the renaturation is performed at protein concentrations that are too high, inactive aggregates are the predominant product. These aggregates are often large and insoluble, and can be removed by low-speed centrifugation. They are, however, sometimes of smaller size, requiring more elaborate separation procedures to obtain the pure, fully active, renatured protein (Murry-Brelier, 1989).

Very little is known about the molecular mechanisms responsible for the formation of aggregates during protein folding. It is generally assumed that the major cause of aggregation is the insolubility of the denatured state. When unfolded, protein molecules have their hydrophobic core exposed to solvent, and aggregation would simply result from nonspecific interactions between hydrophobic regions of different polypeptide chains. Yet, at least in some instances, the interactions leading a given polypeptide chain to aggregate have been shown to be specific, occurring only between identical polypeptide chains, and not with foreign ones (London et al., 1974). Thus, the formation of such specific aggregates might reflect the ability of local complementary intermediate structures (secondary or supersecondary structures, or domains) to form "wrong" interchain interactions rather than the "correct" intrachain interactions which determine the native conformation (London et al., 1974: Goldberg & Zetina, 1980).

It has been proposed that a kinetic competition between these two types of interactions (interchain and intrachain) occurs during the refolding of a protein and that this competition is responsible for the decreased yield of renatured protein as a function of the protein concentration (Zettlmeissl et al., 1979; Orsini & Goldberg, 1978). Indeed, intrachain interactions are essentially unimolecular; their rate of formation should not depend on the concentration. On the other hand, interchain interactions correspond to a multimolecular reaction. The rate is expected to increase rapidly with the concentration. At low protein concentration, aggregation should be slow, and intramolecular interactions leading to the native state would be expected to prevail over aggregation. At high protein concentration, aggregation should be faster than the (first-order) formation of intrachain interactions, resulting in a greatly reduced yield of renatured protein. A similar, but more elaborate, model has been proposed to account for the concentration dependence of both the rate and extent of renaturation during the refolding of bovine growth hormone (Brems, 1988). It involves the existence of an insoluble associated folding conformer that would appear transiently during the renaturation of the protein and that would be the precursor to irreversible aggregates. Both models were originally inferred from the dependence of the final yield on the protein concentration. Though the time range of formation and disappearance of the transient insoluble conformer has been experimentally defined (Brems, 1988), no direct comparison has been made so far between the kinetic properties of the productive and the abortive pathways leading to the native protein on one hand and aggregates on the other. The purpose of the studies reported here was to fill this gap and provide a better test of the model. On the basis of the mechanism presented above, there should be a step in the folding pathway leading to the native state beyond which the protein becomes "committed" to become native. That is, there should be a stage beyond which the activation energy for reversal of the pathway is too high, so that there is no significant reversibility. Indeed, once refolding has been completed, the native protein can be concentrated without aggregation. Similarly, aggregates can be diluted, and no native protein will appear [unless an unfolding–refolding cycle is performed, using a denaturing agent to dissolve the aggregates (London et al., 1974)]. Therefore, there also should be a step on the pathway toward aggregation where the protein becomes "committed" to become aggregated. Characterization of the kinetics of these two "commitment" reactions during the folding of a protein should help in the development of a quantitative understanding of the yield vs concentration profile of that protein.

For this study, we chose a well-characterized protein, egg white lysozyme, the folding of which has been described in detail (Epstein & Goldberger, 1963; Yutani et al., 1968; Saxena & Wetlaufer, 1970; Ristow & Wetlaufer, 1973; Wetlaufer et al., 1974; Anderson & Wetlaufer, 1976; Johnson et al., 1976; Acharya & Taniuchi, 1976). After denaturation under reducing conditions, the enzyme exhibits the characteristic difficulties encountered during renaturation, including the need for reoxidation of disulfide bridges. In the following experiments, we analyze in detail the effect of the initial and final protein concentrations on the final yield of the renaturation process, and describe double-mixing experiments aimed at characterizing the kinetics of commitment toward either the native state or inactive aggregates during the renaturation/reoxidation of denatured–reduced lysozyme.

## Materials and Methods

*Proteins and Chemicals.* Hen egg white lysozyme (40 000 units/mg) was obtained from Boehringer Mannheim, Turkey egg white lysozyme (77 000 units/mg) was from Sigma and bovine serum albumin from Serva.

*Micrococcus lysodeikticus* dried cells were from Sigma; reduced and oxidized glutathione was from Boehringer Mannheim. All other chemicals were reagent grade.

*Enzyme and Protein Assays.* Lysozyme activity 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. The assay volume was 1 mL. One unit of activity corresponds to an absorbance decrease of 0.0026 per minute.

Lysozyme concentrations were determined spectrophotometrically, with the extinction coefficients ($\epsilon_{280nm}^{1mg/mL} = 2.63$ for native lysozyme and 2.37 for denatured lysozyme) reported by Wetlaufer et al. (1974). Serum albumin concentrations were determined by weight upon preparation of solutions.

*Denaturation–Reduction.* Denatured and reduced hen lysozyme, turkey lysozyme, and bovine serum albumin were generated from the pure commercial proteins by a slight modification of the procedure of Ristow and Wetlaufer (1973). Protein solutions (20 mg/mL) in 0.1 M Tris-HCl, pH 8.6, containing 10 M urea and 0.15 M dithioerythritol were incubated for 2 h at 20 °C. The solutions were then acidified to pH 3 by addition of 1 N HCl, dialyzed extensively against 0.1 M acetic acid at 4 °C, and then freeze-dried. The resulting proteins were stored under nitrogen at −20 °C.

Denatured hen lysozyme was carboxymethylated by reaction of denatured and reduced lysozyme in 10 M urea/0.15 M DTE, prepared as above, with 0.4 M iodoacetic acid (i.e., a

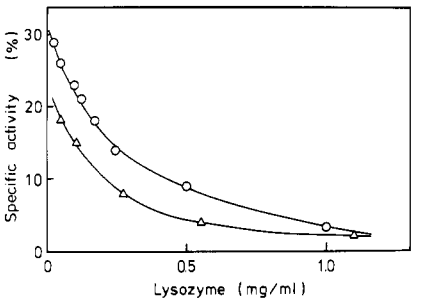



FIGURE 1: Yield of enzymatic activity as a function of lysozyme concentration during refolding. Denatured and reduced hen lysozyme (11 mg/mL) or turkey lysozyme (10 mg/mL) in 0.01 N HCl was diluted 10–400-fold into renaturation buffer at 20 °C by vigorous agitation with a vortex mixer. Before the addition of denatured lysozyme, the renaturation buffer was supplemented with 0.01 N HCl so as to maintain the same 0.001 N HCl concentration during the renaturation in all samples. After overnight incubation, the enzymatic activity was assayed. The yield (in percent) is expressed as the activity recovered relative to the "initial" activity, calculated from the specific activity of the corresponding native lysozyme and the protein concentration in each sample. (Δ) Hen lysozyme, (O) turkey lysozyme.

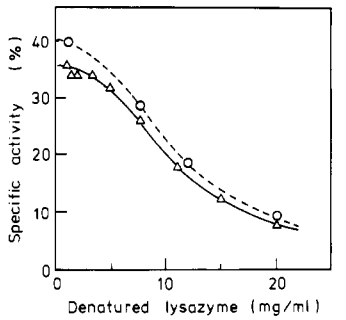



FIGURE 2: Yield of enzymatic activity as a function of the initial concentration of denatured lysozyme. Solutions of denatured–reduced hen or turkey lysozyme in 0.01 N HCl at concentrations ranging from 20 to 0.5 mg/mL were prepared and diluted into renaturation buffer to a final lysozyme concentration of 0.05 mg/mL. Before the addition of denatured lysozyme, the renaturation buffer was supplemented with 0.01 N HCl so as to maintain the same 0.001 N HCl concentration during the renaturation in all the samples. After overnight incubation at 20 °C, the enzymatic activity was assayed; it is expressed as the yield (in percent) relative to the initial activity. (Δ) Hen lysozyme; (O) turkey lysozyme.

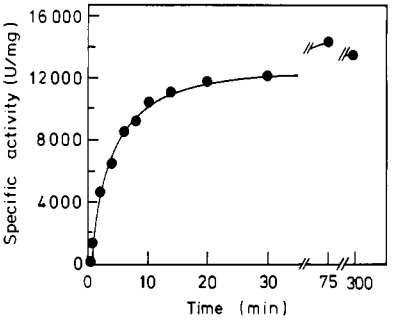



FIGURE 3: Kinetics of reactivation of turkey lysozyme. Denatured and reduced turkey lysozyme (5 μL of a 11.4 mg/mL solution in 0.01 N HCl) was diluted into 495 μL of renaturation buffer at 20 °C. After various times, 10-μL aliquots were withdrawn and immediately assayed in 1 mL of assay mixture at pH 6.2. This procedure was shown (Saxena & Wetlaufer, 1970) to yield valid renaturation kinetics. Though the activity assay lasted 1–2 min, the absorbance varied linearly with time. This indicates that the refolding is effectively blocked in the assay mixture presumably as a result of the low glutathione concentration and the reduced pH. The resulting specific activity is plotted as a function of the renaturation time.

0.1 molar excess over the free SH groups of DTE). After 30-min incubation at room temperature, the solution was acidified, dialyzed, and lyophilized as above. Free SH groups were determined by DTNB titration to be less than 0.1 per carboxymethylated denatured lysozyme molecule.

*Renaturation–Reoxidation.* The reconstitution of native lysozyme was achieved according to Saxena and Wetlaufer (1970). Denatured and reduced lysozyme was dissolved in 0.01 N HCl, then rapidly diluted (at least 10-fold) into renaturation buffer (0.1 M Tris-HCl, pH 8.2, 1 mM EDTA, 3 mM reduced glutathione, and 0.3 mM oxidized glutathione), and incubated at 20 °C for at least 3 h. The enzymatic activity was measured, and the yield of the renaturation process was determined as the total activity obtained after renaturation, relative to that of the native enzyme prior to its denaturation.

The renaturation critically depends on the efficiency of the mixing during the dilution of the protein in the renaturation buffer. Therefore, to ensure reproducible mixing conditions, the desired volume of renaturation buffer was added to an Eppendorf tube, the protein was deposited as a droplet on the wall of the tube, above the buffer meniscus, and mixing was effected by vigorous agitation with a Vortex mixer for 15 s.

*Monoclonal Antibodies.* The monoclonal antibodies D1.3 and F9.13.2, raised against hen egg white lysozyme, were generously supplied by Prof. R. Poljak as ascite fluid. The immunoglobulins were precipitated with 50% saturated ammonium sulfate, then dialyzed, and used without further purification.

## Results

*Concentration Dependence of the Renaturation Yield.* Denatured and reduced lysozyme can be renatured (i.e., refolded and reoxidized) by incubation at slightly alkaline pH in the presence of mixtures of oxidized and reduced glutathione (Saxena & Wetlaufer, 1970), and the yield can reach about 70% under optimal conditions (Wetlaufer et al., 1974). To examine the influence of protein concentration during folding on the yield of active lysozyme, denatured enzyme at about 10 mg/mL was rapidly diluted (between 10- and 200-fold) and vigorously mixed with renaturation buffer. After an overnight incubation at 20 °C, the different dilutions were assayed for lysozyme activity. The results obtained with hen and turkey egg white lysozyme are presented in Figure 1. Though the renaturation yield obtained with turkey lysozyme was, at a given concentration, consistently somewhat higher than that obtained with hen lysozyme, the renaturation of the two enzymes exhibited very similar concentration dependences.

The yields obtained at the lower protein concentrations were significantly lower than those reported earlier by Saxena and Wetlaufer (1970). The major experimental differences between those experiments and ours were the way of performing the dilution, and the concentration of lysozyme in the denatured state. To test the effect of the initial concentration of denatured lysozyme on the yield, several solutions of denatured lysozyme (concentration ranging between 0.5 and 20 mg/mL) were prepared in 0.01 M HCl and diluted to 0.05 mg/mL in renaturation buffer. The results of this experiment, shown in Figure 2, demonstrate that with identical refolding conditions (i.e., 0.05 mg/mL), the yield decreased when the initial denatured protein concentration was increased.

*Kinetics of Renaturation of Lysozyme.* The kinetics of renaturation of hen lysozyme have been reported (Saxena & Wetlaufer, 1970; Wetlaufer et al., 1974). We studied the kinetics of renaturation of turkey lysozyme, and found them to be similar to those of hen lysozyme (Figure 3), with a half-time of reaction of 4.5 min.

*Kinetics of the Commitment to Aggregation.* To determine the rate of appearance of a molecular species that is committed

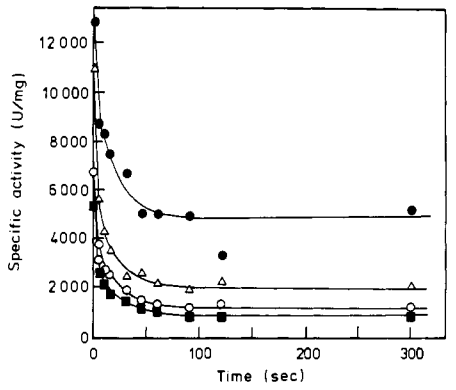



FIGURE 4: Kinetics of the "commitment" to aggregation. Samples of denatured and reduced hen lysozyme (14.8 mg/mL in 0.01 N HCl) were diluted 10- (■), 20- (○), 40- (△), or 80-fold (●) into 95 μL of renaturation buffer at 20 °C. After various times at 20 °C, each sample was diluted further 10-fold with 900 μL of renaturation buffer, incubated overnight at 20 °C, and assayed for lysozyme activity. The specific activity, determined from the enzymatic activity thus obtained and from the protein concentration expected from the various dilutions, is shown for each sample as a function of the time elapsed between the initial and final dilutions in renaturation buffer. Values at time zero were obtained by direct 100-, 200-, 400-, and 800-fold dilutions in renaturation buffer.

to aggregate, a two-step refolding approach was employed. Denatured lysozyme was first diluted into renaturation buffer at "high" protein concentration, incubated for various time intervals, and then diluted again 10-fold with renaturation buffer and incubated overnight to allow the renaturation to go to completion at "low" protein concentration. In such an experiment, the second dilution should permit the uncommitted protein to proceed toward the native state. On the other hand, the protein which irreversibly went onto the aggregation pathway during the initial incubation at high protein concentration should no longer be able to renature, even after the second dilution. The results of such experiments, performed at four different protein concentrations, are shown in Figure 4. From these results, it appears that the commitment to aggregation is a rapid process. One minute after the first dilution, no further aggregation takes place, and, in the hand-mixing experiments (where the shortest incubation time is 5 s), only the end of the kinetics can be observed. Therefore, it seemed inappropriate to undertake a quantitative analysis of the experimental curves to determine the order and the rate constant of the aggregation reaction.

*Kinetics of the Commitment to Renaturation.* An experiment, similar in principle to that just described, has been performed to determine the rate of appearance of a molecular species that is committed to refold into the native conformation. However, instead of incubating first at "high" concentration and then diluting to low concentration, the opposite had to be done. To achieve a rapid jump to higher protein concentration, denatured *turkey* lysozyme was first diluted to low (about 0.1 mg/mL) concentration in renaturation buffer and incubated for various time intervals. Denatured *hen* lysozyme was then rapidly added at high (ca. 1 mg/mL) protein concentration, and the renaturation of both lysozymes was left to proceed to completion by an overnight incubation. The activity of the turkey lysozyme was then specifically determined. For such an experiment, two requirements had to be fulfilled. First, a method had to be found for measuring the turkey lysozyme without interference from the excess hen lysozyme. This appeared possible by using a monoclonal antibody that would inhibit hen, but not turkey, lysozyme. Second, hen and turkey lysozyme should interact at high concentration during renaturation and produce hybrid aggregates. This was very likely in view of the very strong similarities in the sequence (LaRue & Speck, 1970) and folding properties (see Figure 3) of the two molecules.

Table I: Inhibition of Hen and Turkey Egg White Lysozyme by Monoclonal Antibody F.9.13.2[a]

| | hen | | turkey | |
|---|---|---|---|---|
| lysozyme (μL) | 50 | 50 | 50 | 50 |
| mAB (μL) | 0 | 5 | 0 | 5 |
| specific activity (units/mg) | 30500 | <1000 | 75000 | 75000 |

[a] Determination of enzyme activity after incubation of a 42 μg/mL solution of hen or turkey lysozyme with or without mAB.

Table II: Interspecies Aggregate Formation upon Refolding of a Mixture of Hen and Turkey Lysozyme[a]

| | turkey alone | hen alone | turkey + hen |
|---|---|---|---|
| total activity before denaturation[b] (units/mL) | 7700 | 34200 | 41900 |
| total activity after renaturation (units/mL) | 3460 | 690 | 960 |
| turkey lysozyme activity before denaturation[b] (units/mL) | 7700 | 0 | 7700 |
| turkey lysozyme activity after renaturation[c] (units/mL) | 3450 | 7 | 210 |
| yield of renatured turkey lysozyme[d] (%) | 45 | | 2.7 |

[a] Solutions containing denatured and reduced lysozyme in 0.01 M HCl were prepared to the following concentrations: turkey lysozyme alone, 1.0 mg/mL; hen lysozyme alone, 8.55 mg/mL; mixture of turkey and hen lysozymes, 1.0 and 8.55 mg/mL, respectively. To 180 μL of renaturation buffer was added 20 μL of denatured lysozyme with vigorous vortex mixing. After overnight incubation, the samples were submitted to a 10-min centrifugation at 10000 rpm in an Eppendorf centrifuge. To 20-μL samples of each supernatent was added 10 μL of mAB, and the residual activity was measured. The activity in the absence of mAB was also determined. [b] Activities before denaturation correspond to the initial activities of the native lysozyme molecules used in each denaturation–renaturation experiment. They were calculated from the hen and turkey lysozyme concentrations in each final renaturation mixture and the initial specific activities of the lysozyme preparations (see Materials and Methods). [c] Turkey lysozyme activity was determined by measuring the enzymatic activity of renatured solutions in the presence of mAB. [d] The yield was obtained from the activity determined in the presence of monoclonal antibody as compared to the turkey lysozyme activity before denaturation.

We therefore first searched for an antibody that would strongly inhibit hen lysozyme but not turkey lysozyme. Two monoclonal antibodies, D1.3 and F9.13.2, that bind tightly to hen lysozyme but not the turkey enzyme were tested (Harper et al., 1987). While D1.3 inhibited the hen lysozyme only by 70%, the inactivation by F9.13.2 was nearly complete. Under the same conditions, the activity of turkey lysozyme was unaffected (Table I). Thus, using the latter antibody, it was possible to specifically measure the activity of turkey lysozyme in a mixture with hen lysozyme.

Next, the ability of hen and turkey lysozyme to form hybrid aggregates during refolding at high concentration was investigated. Turkey and hen lysozymes were diluted in renaturation buffer (final concentrations 0.10 and 0.85 mg/mL, respectively), either separately or together as a mixture. After overnight incubation to achieve renaturation, the precipitated protein was removed by centrifugation (to eliminate aggregates that might still be immunoreactive), and the lysozyme activity was determined after addition of the monoclonal antibody.

Table II shows the results thus obtained. While turkey lysozyme alone at 0.1 mg/mL renatured with high yield (45%), the presence of hen lysozyme strongly interfered with the renaturation of turkey lysozyme. Indeed, the yield of active turkey lysozyme was reduced to 2.7% in the presence of 0.85

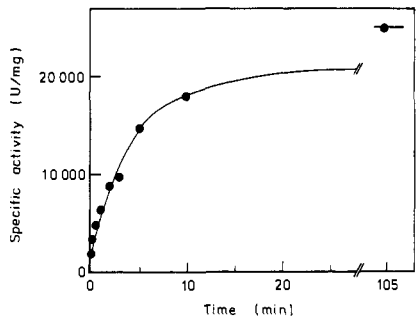



FIGURE 5: Kinetics of the "commitment" to renaturation. Ten microliters of denatured and reduced turkey lysozyme (1.05 mg/mL in 0.01 N HCl) was diluted in 80 μL of renaturation buffer. After various times of incubation at 20 °C, 10 μL of denatured and reduced hen lysozyme (9.1 mg/mL in 0.01 N HCl) was added to each sample. After 2-h incubation at 20 °C, the samples were centrifuged for 10 min at 10000 rpm in an Eppendorf centrifuge. Twenty microliters of supernatant was mixed with 10 μL of immunoglobulin F9.13.2 (8.4 mg/mL), incubated for 5 min at room temperature, and assayed for lysozyme activity. The specific activity is plotted as a function of the time elapsed between the initiation of the turkey lysozyme renaturation and the addition of hen lysozyme. The value at time zero was obtained by first mixing turkey and hen lysozyme in 0.01 N HCl and then diluting the mixture in renaturation buffer. In a control experiment with no hen lysozyme added, a specific activity of 24400 units/mg was obtained.

mg/mL hen lysozyme. Thus, at a total (turkey + hen) lysozyme concentration of 0.95 mg/mL, the yield was very similar to that found when turkey lysozyme alone was renatured at 1 mg/mL (see Figure 1). This confirmed that hybrid aggregates were indeed formed during the refolding of turkey and hen lysozyme mixtures and that the interactions responsible for the aggregation of hen and/or turkey lysozymes were the same (or at least very similar).

The kinetics of commitment to the native state could therefore be measured as outlined above. The results of such an experiment are depicted in Figure 5. The commitment to renaturation was a comparatively slow process, with a half-time of about 4.5 min, i.e. identical, within the experimental precision, with the half-time of the renaturation itself.

*Nature of the Interactions Leading to Aggregation.* From the results described in Figure 4, it might have been inferred that the aggregation reaction is fast and can occur only when the protein is in a short-lived transient state that no longer exists after 0.5–1 min. This might have suggested that the species prone to aggregation correspond to largely unfolded molecules, with their hydrophobic core still exposed to the solvent. However, the results in Figure 5 show that lysozyme remained sensitive to aggregation (in the presence of an excess of denatured molecules) for a much longer period of time, at a stage where the molecules were probably at least partly folded and their hydrophobic core shielded from the solvent. It therefore seemed of interest to investigate the nature of the interactions responsible for the formation of aggregates.

First, to decide whether or not incorrect interchain disulfide bonds were involved in the formation of aggregates, hen lysozyme was unfolded and reduced, and its sulfhydryl groups were blocked by reaction with iodoacetic acid.

A mixture of denatured–carboxymethylated hen lysozyme (10 mg/mL) and denatured turkey lysozyme (1.1 mg/mL) in 0.01 M HCl was diluted 10-fold in renaturation buffer and incubated for 3 h at 20 °C. The total lysozyme activity was then measured and found to be 350 units/mg of turkey lysozyme. In parallel experiments, denatured turkey lysozyme alone (1.1 mg/mL) or carboxymethylated hen lysozyme alone (10 mg/mL) was submitted to the same renaturation process. The activity recovered was 28000 units/mg for the turkey lysozyme. As expected, no activity could be detected for the carboxymethylated hen lysozyme. These results show that in the mixture, the carboxymethylated protein indeed formed abortive aggregates with turkey lysozyme, thus demonstrating that free SH groups were not needed to form aggregates and that wrong disulfides were not the major cause of aggregates.

The next question we asked was whether or not the interactions responsible for the formation of aggregates are specific. To answer that question, a mixture of denatured–reduced bovine serum albumin (10 mg/mL) and denatured–reduced turkey lysozyme (1.1 mg/mL) in 0.01 M HCl was diluted 10-fold in renaturation buffer, incubated for 3 h at 20 °C, and centrifuged at 10000 rpm for 10 min. The supernatant was then assayed for lysozyme activity. No enzymatic activity could be detected, thus demonstrating that nonspecific interactions entrapped lysozyme molecules in heterologous aggregates with serum albumin. It therefore could be concluded that nonspecific interactions were involved in the aggregation reaction.

There was a significant difference in behavior between reduced lysozyme and BSA or carboxymethylated lysozyme. Indeed, when denatured–reduced hen lysozyme was diluted into renaturation buffer containing *native* turkey lysozyme, the turkey lysozyme was not affected. After centrifugation and addition of monoclonal antibody F9.13.2, the enzymatic activity of turkey lysozyme was identical with that determined before addition of hen lysozyme. On the other hand, addition of denatured carboxymethylated hen lysozyme (1 mg/mL) or denatured–reduced BSA (0.95 mg/mL) to renaturation buffer containing 0.1 mg/mL *native* turkey lysozyme resulted in the coprecipitation of about 60% of the native turkey lysozyme. Furthermore, a vigorous vortex mixing of the renaturation mixtures before centrifugation resulted in the release into solution of most of the coprecipitated turkey lysozyme. When the same vortex treatment was applied to the aggregates formed during the renaturation of reduced lysozyme, no active molecules were released into solution. Thus, the nonspecific interactions responsible for the coprecipitation appeared to be much weaker than the interactions responsible for aggregation.

Finally, to decide whether denatured carboxymethylated lysozyme and BSA decrease the yield of renatured–reduced turkey lysozyme only through coprecipitation, or also through irreversible aggregate formation, a delayed mixing experiment similar to that depicted in Figure 5 was performed. This time, denatured and reduced turkey lysozyme was first diluted to 0.1 mg/mL in renaturation buffer. At various times after dilution, either denatured BSA (to 0.95 mg/mL) or denatured–carboxymethylated hen lysozyme (1.1 mg/mL) was added. After incubation overnight to allow for complete renaturation, the lysozyme activity was assayed. Except for the lower yield due to coprecipitation, the kinetics were similar to those in Figure 5. Thus, both BSA and carboxymethylated lysozyme appear to prevent the proper refolding of reduced turkey lysozyme through a mechanism involving the same type of interactions that lead to aggregate formation during the renaturation of concentrated lysozyme.

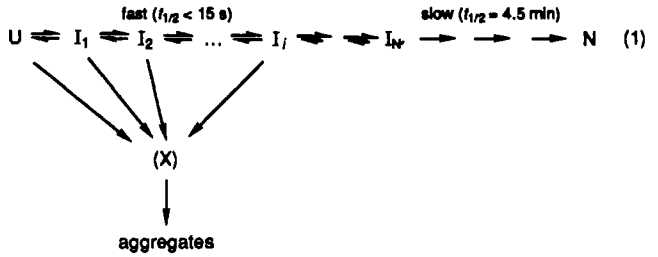
## Discussion

The results described above have confirmed that both hen and turkey egg white lysozymes, like many other proteins, can be renatured with high yields only at low protein concentration. With increasing concentration, the yield of renatured protein decreases, reaching only a few percent at concentrations above 100 μM. The yields obtained in the present study were, even at low protein concentration (i.e., below 10 μM), somewhat

lower (about 35%) than those (70–80%) reported earlier (Wetlaufer et al., 1974; Saxena & Wetlaufer, 1970). The difference is not likely to be due to the lower temperature (20 °C) at which the present renaturation studies were performed, since the yield of renatured lysozyme does not depend strongly on the temperature (Saxena & Wetlaufer, 1970; Wetlaufer et al., 1974). Rather, it is likely to reflect the state of the starting lysozyme preparations we used, which had much higher specific activity than those used in the earlier studies. Therefore, though the specific activities we obtained for renatured hen lysozyme were similar to, or even higher than, those previously reported in the literature, the yields may appear lower. Apart from the protein concentration in the process of reconstitution, the yield was also shown to depend on the lysozyme concentration in the denatured state, e.g., in 0.01 N HCl. Two interpretations can account for this observation. One would be that the denatured–reduced lysozyme molecules undergo an association–dissociation equilibrium in acid. Such concentration-dependent association of acid-denatured polypeptide chains has been described for several proteins (Rudolph & Jaenicke, 1976; Murry-Brelier & Goldberg, 1988; Goto & Fink, 1989). However, for those proteins, the association in acid is rather slow, and not reversible, while no irreversibility was observed in the case of the denatured–reduced lysozyme. An alternate interpretation of the observed dependence of the yield on the denatured lysozyme concentration is related to the fate of the protein during the very early phases of the dilution procedure used to initiate the renaturation reaction. In the present case, raising the pH with the strongly buffered renaturation medium can be considered as "instantaneous". On the other hand, dispersing the concentrated protein in the renaturation buffer may require a nonnegligible time, as compared to the half-time of aggregation. Thus, a sufficiently high local protein concentration may exist transiently, thereby allowing some aggregation to take place. In this case, a higher initial concentration would result in a higher transient concentration and hence in more aggregates and a lower yield of renatured protein. This interpretation seems plausible in view of the often encountered strong dependence of the yield on the procedure used to dilute the denatured protein into the renaturation buffer. This has been well documented, e.g., in the case of the renaturation of the unfolded $\beta_2$ subunits of *Escherichia coli* tryptophan synthase which, under otherwise identical renaturation conditions, refold with yields ranging between 5% and 95% depending on the efficiency of the agitation upon initiating the renaturation process (Gaucher, 1987). Moreover, when one tries (data not shown) to repeat the experiments depicted in Figure 4 by using a 3-syringe-2 mixer stopped-flow apparatus, the yield obtained at 1 mg/mL lysozyme was unexpectedly high (about 20%) compared to the above-mentioned mixing experiments, thus showing again that processes occurring during the mixing phase are crucial for the final yield of the renaturation reaction. This is in no way surprising in view of the kinetics observed for the "commitment" of lysozyme to aggregate. Indeed, the present double dilution experiments clearly demonstrate that the commitment to aggregate is a very fast reaction. This could be expected since aggregation is known to be a fast process of higher than second order (Zettlmeissl et al., 1979), and since the commitment has obviously either to precede or to coincide with the aggregation itself.

Up to this point, the behavior of lysozyme during its renaturation seems to confirm current views on the kinetic competition between renaturation and aggregation during the in vitro folding of proteins. However, there are some unexpected properties of this mechanism which have not been considered before. Most obvious, the kinetics of commitment to refold into the native state were found to be much slower than the commitment toward aggregation, even under conditions where the refolding leads to reasonably high renaturation yields. According to a simple kinetic competition between a pathway leading to the native state and a pathway leading to inactive aggregates, this should have caused very low renaturation yields. Yet, reasonably high renaturation yields (about 20%) were obtained even under conditions (0.185 mg/mL lysozyme) where the commitment to refold correctly had a half-reaction time of 4.5 min as compared to less than 30 s for the commitment to aggregate. The folding vs aggregation competition therefore seems more complex than originally thought.

This could also be infered from the kinetics depicted in Figure 4. At the four concentrations investigated, the observable parts of the kinetics seem to proceed at comparable rates, and to level off and terminate after about 1 min. Indeed, no further decrease in the yield could be detected when the protein was incubated for more than 1 min between the two successive dilutions into renaturation buffer. At first sight, this could be interpreted as an indication that the commitment to aggregation is a high-order reaction. The large concentration dependence of the reaction rate would explain why the aggegation abruptly stops when the residual concentration of protein which is not aggregated becomes low. However, the final concentration of unaggregated protein present 1 min after the initial dilution is 2-fold higher when the renaturation of hen lysozyme is performed at 1.48 mg/mL compared to 0.37 mg/mL (1.480 vs 740 units/mL, respectively). That the observable part of the commitment kinetics proceeds with similar rates, and ultimately stops, at these 2-fold different concentrations of unaggregated protein strongly suggests that the commitment to refold into the native conformation has already occurred within the first minute of the folding process, thus preventing further aggregation during later phases of the folding process. This conclusion apparently contradicts the results depicted in Figure 5, which show that the irreversible commitment to the native state occurs much more slowly, with a half-reaction time of 4.5 min. Two distinct models permit the solution of this paradox. The first model assumes that the aggregation involves two complementary association areas located in distinct regions of the polypeptide chain. Aggregation would result from the interaction of one area of one chain with the complementary area of another chain. To prevent aggregation, it then would suffice to bury one of these interaction areas in the interior of the folding protein. Thus, if the burying of one of the areas is completed (for lysozyme after 1 min), the partially folded molecules will no longer aggregate, even though their second aggregation area is still exposed. However, if new, unfolded, molecules with their first area still exposed are added to the partially folded chains, interchain interactions would occur, leading to "late" aggegation as illustrated in Figure 5. In such a mechanism, the interactions between "complementary" association areas would have to be, at least to some extent, stereospecific. Because nonspecific aggregation (with serum albumin for instance) does occur even at late stages of lysozyme folding, such a mechanism based on stereospecific complementary interaction areas appears unlikely. A second, more plausible, model relies on equilibria coupled with irreversible kinetic processes. We shall assume that folding of lysozyme proceeds through a set of intermediate states, appearing in time in the order shown in eq 1. We shall also assume that all states U to $I_i$ can either irreversibly enter the aggregation pathway (via a common




intermediate, x?) or proceed to the native state, while none of the states after $I_i$ can aggregate. Finally, we shall assume that all steps between $I_i$ and $I_N$ occur very rapidly and are reversible with an equilibrium constant much in favor of the more folded state, while all states beyond $I_N$, appear slowly ($t_{1/2}$ = 4.5 min) and irreversibly under the folding conditions used. Let us now consider the fate of a molecule that would fold according to such a scheme. During the early phases of the folding process, the total concentration of U, $I_1$, ..., $I_i$ would be transiently high enough to favor rapid aggregation. Simultaneously, some molecules would form the intermediates $I_i$ to finally reach $I_N$, thus escaping aggregation. If reaching $I_N$ were indeed rapid (less than 1 min), and if the equilibria were much in favor of $I_N$, then after 1 min the total concentration of U, $I_1$, ..., $I_i$ would be very low so that their concentration-dependent aggregation would no longer be observed. If, however, one were to increase artificially the concentration of U, $I_1$, ..., $I_i$ by adding new unfolded molecules (e.g., denatured hen lysozyme in the experiment of Figure 5), then aggregates would be formed rapidly with the preexisting U to $I_i$ (turkey lysozyme intermediates). Because the preexisting intermediates preceding $I_N$ are in rapid equilibrium, this would result in shifting back the overall population toward aggregation. This model satisfactorily accounts for the originally surprising observation that the irreversible commitment toward the native state occurs much later than the end of the aggregation during the refolding of lysozyme.

In conclusion, folding of lysozyme is suggested to proceed according to eq 2 which summarizes the essential features of the above model. In eq 2, three classes of intermediates are

$$U \rightleftharpoons A \rightleftharpoons B \xrightarrow{t_{1/2} = 4.5 \text{ min}} C \rightarrow N \qquad (2)$$

depicted: A and B as "open" and "closed" intermediates which form rapidly and reversibly, and do (A) or do not (B) form aggregates, and C, a class of "native like" intermediates, which form slowly and irreversibly, and do not aggregate.

One might attempt to correlate those three classes of intermediates to states that have been shown to exist at different stages of protein folding. It has been shown, mainly by Kuwajima and his co-workers, that an initial extremely rapid step in protein folding (less than 10-ms half-time) consists of the formation of secondary structure (Kuwajima et al., 1987; Labhardt, 1986; Kuwajima, 1989). Next, with a half-time of 50–200 ms, a "molten globule" is formed (Ptitsyn, 1973; Semisotnov et al., 1987; Kuwajima, 1989; Ptitsyn et al., 1990). Then, much more slowly, tight packing of the side chains within the molten globule results in the native, stable (i.e., nearly irreversible under "folding" conditions) conformation. It is tempting to identify class A with the early intermediates exhibiting secondary structure but no tertiary fold and thus having their hydrophobic residues exposed for aggregation. Class B may be assumed to correspond to the unstable "molten globule" (with the hydrophobic residues already in a hydrophobic core and thus protected from aggregation). Class C would result from the slow tight packing of side chains into a stable folded state which either is native or is close to native. The concentration dependence of the yield would then result from a kinetic competition between the aggregation of intermediates from class A and their conversion to intermediates from class B. If the structural characteristics proposed above for the three classes of intermediates were correct, the aggregation would involve essentially the very early folding intermediates that have not yet built up the hydrophobic core of a molten globule. The kinetic competition would be between aggregation and formation of a hydrophobic core. This is in agreement with our observation that aggregation occurs very early in the folding process, and through nonspecific, probably hydrophobic interactions similar to those proposed by Ptitsyn (1973) as responsible for the (poor) stability of the molten globule.

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# Reaction of Proteinases with $\alpha_2$-Macroglobulin: Rapid-Kinetic Evidence for a Conformational Rearrangement of the Initial $\alpha_2$-Macroglobulin–Trypsin Complex†

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ABSTRACT: The kinetics of the reaction of trypsin with $\alpha_2$M were examined under pseudo-first-order conditions with excess inhibitor. Initial studies indicated that the fluorescent dye TNS is a suitable probe for monitoring the reaction over a wide concentration range of reactants. Titration experiments showed that the conformational changes associated with the binding of trypsin to $\alpha_2$M result in an increased affinity of the inhibitor for TNS. Two distinct phases were observed when this dye was used to monitor the progress of the reaction. Approximately half of the fluorescence signal was generated during a rapid phase, with the remainder generated during a second, slower phase. The observed pseudo-first-order rate constant of the first phase varied linearly with the concentration of $\alpha_2$M up to the highest concentration of inhibitor used, whereas the rate constant of the second phase was independent of $\alpha_2$M concentration. The data fit a mechanism in which the association of trypsin with $\alpha_2$M occurs in two consecutive, essentially irreversible steps, both leading to alterations in TNS fluorescence. The initial association occurs with a second-order rate constant of $(1.0 \pm 0.1) \times 10^7$ $M^{-1}$ $s^{-1}$ and is followed by a slower, intramolecular conformational rearrangement of the initial complex with a rate constant of $1.4 \pm 0.2$ $s^{-1}$. The data are consistent with a previously proposed model for the reaction of proteinases with $\alpha_2$M [Larsson et al. (1989) *Biochemistry 28*, 7636–7643]. In this model, once an initial 1:1 $\alpha_2$M–proteinase complex forms, the complex either can react with a second proteinase molecule or can undergo a conformational rearrangement that generates a complex greatly reduced in its ability to bind additional proteinase. The detection of two kinetic phases in the present study and the excellent agreement between the range of 1–2 $s^{-1}$ predicted from modeling experiments for the magnitude of this conformational change and the value of 1.4 $s^{-1}$ obtained for the slow phase provide evidence supporting this model.

$\alpha_2$-Macroglobulin ($\alpha_2$M)[1] is a plasma glycoprotein that is capable of reacting with numerous proteinases (Barrett, 1981). This molecule is one member of a family of proteins that contain thiol ester bonds (Sottrup-Jensen et al., 1985). Reaction of $\alpha_2$M with proteinases is initiated by a selective cleavage (Harpel, 1973) at a specific "bait" region of the $\alpha_2$M subunit, which activates the molecule by inducing a series of conformational changes within $\alpha_2$M that can be detected by a variety of physical techniques (Björk & Fish, 1982; Gonias et al., 1982; Strickland & Bhattacharya, 1984; Strickland et al., 1984). These conformational alterations result in activation of the thiol ester bonds which increases the reactivity of these bonds toward nucleophilic molecules (Sottrup-Jensen et al., 1981), in a reduction of the activity of the proteinase toward large macromolecular substrates (Barrett & Starkey, 1973), and in the generation of regions on $\alpha_2$M (Imber & Pizzo, 1981; Kaplan & Nielsen, 1979) that interact with a specific cell surface receptor. This receptor has been recently identified

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[1] Abbreviations: $\alpha_2$M, $\alpha_2$-macroglobulin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid.

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