Oxidative Renaturation of Lysozyme at High Concentrations

Diane L. Hevehan, Eliana De Bernardez Clark

Department of Chemical Engineering, Tufts University, Medford, Massachusetts 02155; telephone: 617-628-5000 x2573; fax: 617-627-3991
e-mail: edeberna@pearl.tufts.edu

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Abstract: Newly synthesized cloned gene proteins expressed in bacteria frequently accumulate in insoluble aggregates or inclusion bodies. Active protein can be recovered by solubilization of inclusion bodies followed by renaturation of the solubilized (unfolded) protein. The recovery of active protein is highly dependent on the renaturation conditions chosen. The renaturation process is generally conducted at low protein concentrations (0.01–0.2 mg/mL) to avoid aggregation. We have investigated the potential of successfully refolding reduced and denatured hen egg white lysozyme at high concentrations (1 and 5 mg/mL). By varying the composition of the renaturation media, optimum conditions which kinetically favor proper folding over inactivation were found. Solubilizing agents such as guanidinium chloride (GdmCl) and folding aids such as L-arginine present in low concentrations during refolding effectively enhanced renaturation yields by suppressing aggregation resulting in reactivation yields as high as 95%. Quantitatively the kinetic competition between lysozyme folding and aggregation can be described using first-order kinetics for the renaturation reaction and third-order kinetics for the overall aggregation pathway. The rate constants for both reactions have been found to be strongly dependent on denaturant and thiol concentration. This strategy supercedes the necessity to reactivate proteins at low concentrations using large renaturation volumes. The marked increase in volumetric productivity makes this a viable option for recovering biologically active protein efficiently and in high yield in vitro from proteins produced as inclusion bodies within microbial cells. *Biotechnol Bioeng* **54**: 221–230, 1997.
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INTRODUCTION

Newly synthesized proteins expressed from cloned genes frequently accumulate as inactive aggregates with only a small portion actually reaching native and active conformation. Two routes have been followed to improve production of active recombinant proteins. One method is to optimize levels of properly folded proteins during the initial in vivo folding step within the bacterial cell. The more common technique, however, involves developing methods to increase recovery of native protein during the in vitro folding process. The environment in which a protein folds may be engineered to provide increased stability or activity. As a protein folds from the denatured state, it proceeds through conformations which lead to the correctly folded structure, including native disulfide bonds. Depending on the conditions during in vitro refolding, there may be a discrepancy between the number of native molecules and the number of originally unfolded protein molecules. This is commonly due to aggregation in which partially folded kinetic intermediates associate incorrectly, rendering the protein insoluble and inactive (Goldberg et al., 1991; Mitraki and King, 1989; Wetzel, 1994). In vitro aggregation, observed as an off-pathway reaction, is closely related to inclusion body formation in vivo and competes with the proper folding reaction (Kiefhaber et al., 1991; Zettlmeiβl et al., 1979).

No general rules exist for generating high yields, but since low recovery of correctly folded protein is often due to aggregation, research has been targeted toward preventing this unproductive side reaction. Protein concentration is believed to be the predominant factor governing aggregation. In the kinetic competition between folding and aggregation, the folding process is a first-order reaction and its conversion is independent of protein concentration, whereas the aggregation pathway is an intermolecular reaction involving at least two polypeptide chains and its rate increases accordingly to some power (≥ 2) of the protein concentration (Kiefhaber et al., 1991; Zettlmeiβl et al., 1979). Folding intermediates tend to aggregate at high concentrations through interactions between normally unexposed hydrophobic regions on their surface. Thus, renaturation yields are a strong function of the initial concentration of denatured protein. Previous studies on reactivation of denatured lactic dehydrogenase (Zettlmeiβl et al., 1979) and lysozyme (Goldberg et al., 1991) demonstrate this relationship. Significant losses in yield due to aggregation were observed as the protein concentration increased in each case. The most direct means of minimizing aggregation is by decreasing protein concentration. It has been suggested that optimum recovery yields can be expected if the protein concentration is lowered to less than 20 μg/mL (Jaenicke and Rudolph, 1989). However, combating aggregation by renaturation at low protein concentra-

Correspondence to: E. De Bernardez Clark

tions is not a cost-effective solution on an industrial scale. Large volumes of renaturation buffer would be necessary to process the desired amount of denatured protein, thereby driving production costs upward beyond economic feasibility.

In this investigation we have explored methods used previously to promote proper folding at low concentrations (<0.1 mg/mL) for their ability to prevent aggregation at higher protein concentrations (≥1 mg/mL). By controlling the environment in which a protein folds, we have designed a procedure allowing for successful renaturation of proteins at high concentrations, eliminating the customary demand for large renaturation buffer volumes to produce functional protein. In particular, addition of solubilizing agents in nondenaturing concentrations to the renaturation buffer seemed to be most effective at decelerating the rate of aggregation. L-Arginine was first found to enhance human tissue-type plasminogen activator renaturation yields (Rudolph, 1990) and has been subsequently used as a folding aid in the reactivation of Fab antibody fragments (Buchner and Rudolph, 1991), single-chain immunotoxins (Buchner et al., 1992), and interleukin-6 receptor (Stoyan et al., 1993). In addition to L-arginine, low concentrations of urea or guanidinium chloride (GdmCl) present in the renaturation buffer can also improve yields (Maeda et al., 1995; Orsini and Goldberg, 1978; Vandenbroeck et al., 1993). Jaenicke and Rudolph (1989) suggest that aggregation is limited by solubilizing agents because they prevent hydrophobic interactions between intermediates. Using denaturing additives to renature highly concentrated proteins offers benefits beyond increasing volumetric productivity. The refolding process is simplified as it is no longer necessary to remove the GdmCl or urea used to denature the protein. Renaturation can be initiated through a direct dilution of the denatured protein and denaturing agents into the desired buffer. In this single-stage approach the concentration of the denaturant is reduced to a level sufficient to induce folding along the productive pathway.

We used hen egg white lysozyme (HEWL) as our model system to search for conditions that can allow oxidative renaturation of proteins at high concentrations. Lysozyme is an excellent model system because its three-dimensional structure is well known; it contains common features to many globular proteins, including four disulfide bonds; its oxidative renaturation has been widely studied (Achayara and Taniuchi, 1982; Epstein and Goldberger, 1963; Fischer et al., 1992, 1993; Goldberg et al., 1991; Rudolph and Fischer, 1990; Saxena and Wetlaufer, 1970; Wetlaufer et al., 1974) and can be monitored using a well-established activity assay; and it has been found to aggregate upon folding at high concentrations (Fischer et al., 1992; Goldberg et al., 1991). The highest single-step lysozyme renaturation yield reported in the literature has been 85% (Saxena and Wetlaufer, 1970), obtained at very low concentrations (0.014 mg/mL). Lysozyme renaturation yields have been improved by utilizing continuous or discontinuous step addition of the denatured protein to the refolding buffer (Fischer et al., 1992; Rudolph and Fischer, 1990). This requires the removal of the denaturant and reducing agents from the unfolded protein by buffer exchange into a low pH acid solution and thus introducing an additional processing step. More recently, Maeda et al. (1995) report high lysozyme renaturation yields obtained by dialyzing denatured lysozyme against a buffer solution of decreasing urea concentration. Although they report high renaturation yields for lysozyme concentrations of up to 5 mg/mL, their protocol requires long renaturation times and might be difficult to implement in large scale. In this study we show that lysozyme can be renatured at high concentrations by rapid dilution of the unfolded protein into renaturation buffer containing nondenaturing concentrations of guanidinium chloride. The proposed method does not introduce additional buffer exchange steps, since removal of the denaturant and reducing agents before renaturation is not necessary.

MATERIALS AND METHODS

Materials

Hen egg white lysozyme (lot no. 111H7010), three-times crystallized, dialyzed, and lyophilized, was obtained from Sigma Chemical Co. Dithiothreitol (DTT) and *Micrococcus lysodeikticus* cells were also from Sigma. Solutions of reduced DTT were prepared immediately prior to each experiment to minimize air oxidation. To avoid artifacts, GdmCl of the ultrapure quality was purchased from ICN Biomedicals Inc. In preparing 8 *M* GdmCl solutions, light heat and agitation were necessary to dissolve the salt. High-performance liquid chromatography (HPLC) grade oxidized glutathione (GSSG) was purchased from Calbiochem-Novabiochem Co. All other chemicals were reagent grade.

Denaturation/Reduction

Lysozyme at 16–40 mg/mL was denatured and reduced in a solution of 8 *M* GdmCl containing 16–32 m*M* DTT, 50 m*M* Tris–HCl, and 1 m*M* ethylenediaminetetraacetic acid (EDTA), adjusted to an apparent pH of 8.0. The resulting protein solutions were incubated for 1 h at 37°C. After reaching room temperature, concentrations of denatured lysozyme were determined spectrophotometrically as described below. In all experiments conducted reduced and denatured lysozyme contained approximately eight free cysteines as determined using Ellman's reagent (Darby and Creighton, 1995).

Renaturation/Reoxidation

To achieve refolding of native lysozyme, a method similar to that described by Saxena and Wetlaufer (1970)

was employed. Following denaturation and reduction, renaturation was initiated by a rapid 8-fold or 16-fold dilution of the denatured lysozyme into renaturation buffer to yield a final concentration of 1–5 mg/mL. Upon investigating the influence of various GdmCl concentrations on reactivation, it was preferred to maintain identical denaturing conditions. Consequently, the renaturation buffer was supplemented with the appropriate amounts of GdmCl to yield an adequate range for analysis. Denatured lysozyme was added to the renaturation buffer and agitated vigorously to avoid creating transient areas of high protein concentration in which the protein would be more susceptible to aggregation. The renaturation media contained 50 m*M* Tris–HCl, 1 m*M* EDTA, and the desired amount of GSSG at 25°C and pH 8.0. Addition of GSSG's reducing partner, GSH, to the renaturation system was not necessary due to the DTT carried over from the denatured solution. In a typical experiment, the refolding solution contained 5 m*M* GSSG and 2 m*M* DTT, resulting in a glutathione ratio [GSH]/[GSSG] of 1.33/1. L-Arginine or GdmCl was added as necessary to the renaturation buffer prior to diluting the denatured protein into the buffer. The refolding solution was incubated at room temperature and samples were withdrawn at the desired times. Lysozyme renaturation kinetics and yields were determined through its enzymatic activity assay.

Quenching

The kinetics of reactivation of lysozyme was monitored by removing 500-μL samples of refolding lysozyme at specific time intervals and quenching the reaction with 50 μL of 0.5 *M* iodoacetic acid. Stopping the refolding reaction prior to dilution and enzymatic analysis provided an accurate method for determining instantaneous activities and overall yield in the timed experiments. The concentrated iodoacetic acid solution was prepared fresh for each experiment by adding the solid to equal volumes of 1 *M* potassium hydroxide and 1 *M* Tris–HCl, pH 7 (Darby and Creighton, 1995) and was used within 90 min after preparation. The unused portion was stored in the dark at −20°C for use with later samples. The activities of the quenched samples, which were kept at −20°C, remained stable for at least up to 1 week. The quenching procedure had no effect on the activity of native lysozyme.

Enzyme Assay

The lysozyme activity assay used was a modification of the method used by Jolles (1962). Dried *M. lysodeikticus* cells were prepared in a 0.067 *M* potassium phosphate buffer suspension, pH 6.2, at a concentration of 0.15 mg/mL. The quenched sample was diluted into 50 m*M* Tris–HCl and 1 m*M* EDTA at pH 8.0 as necessary to yield a final protein concentration of approximately 1.96 μg/mL in the assay mixture. The activity assay was initiated by adding the diluted quenched refolding sample to 3 mL of the cell suspension at 25°C. The decrease in absorbance at 450 nm of the resulting suspension was monitored every 5 s from 30 to 90 s after mixing as the bacterial cell walls were lysed by the enzyme. Absorbance measurements were conducted with a Hewlett Packard HP8452A UV/Vis photodiode array spectrophotometer. A plot of the change in absorbance versus time yielded a linear relationship. By comparing the slope of this line to that obtained using a reference native lysozyme solution of identical protein concentration, lysozyme activities recovered during the reconstitution experiments were expressed as percentage values. Measurements of total protein, soluble protein, and aggregated protein concentrations after full renaturation was allowed to occurred indicated that all the protein that remained soluble was active. The *M. lysodeikticus* cell suspension remained stable for approximately 1 h. Any changes, especially in the cell suspension, became evident by measuring the activity of the reference lysozyme solution at both the start and end of each batch of cell suspension prepared.

Reference assay solutions of lysozyme were prepared by weighing the powdered solid and dissolving it in a known volume of 50 m*M* Tris–HCl and 1 m*M* EDTA buffer at pH 8.0. In order to attain the desired concentration of reference solution, an additional 10% of protein was weighed out. Solid lysozyme is known to bind approximately 10% water. To avoid error in estimating protein content, concentrations of lysozyme in solution were determined by measuring the absorbance at 280 nm using extinction coefficients of 2.63 and 2.37 (cm mg/mL)$^{-1}$ for native and denatured lysozyme, respectively (Wetlaufer et al., 1974). Absorbance measurements were conducted with a Hewlett Packard 8452A UV/Vis photodiode array spectrophotometer.

Aggregation

Turbidity measurements provided a means to monitor formation of aggregates of lysozyme. During and after renaturation, undiluted protein samples were analyzed for light scattering at 600 nm using a Hewlett Packard 8452A photodiode array spectrophotometer. In experiments designed to measure the amounts of soluble and aggregated protein after renaturation is allowed to proceed, reduced-denatured lysozyme (in 8 *M* GdmCl, various concentrations of DTT, 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 8) was rapidly diluted in renaturation buffer containing various amounts of GSSG, 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 8, and allowed to fold for 1 h. At the end of this period, soluble and aggregated protein were separated by centrifugation. The concentration of soluble protein was determined by measuring absorbance at 280 nm (see above). Aggregates were washed twice with TE buffer (50 m*M* Tris–HCl, 1 m*M* EDTA, pH

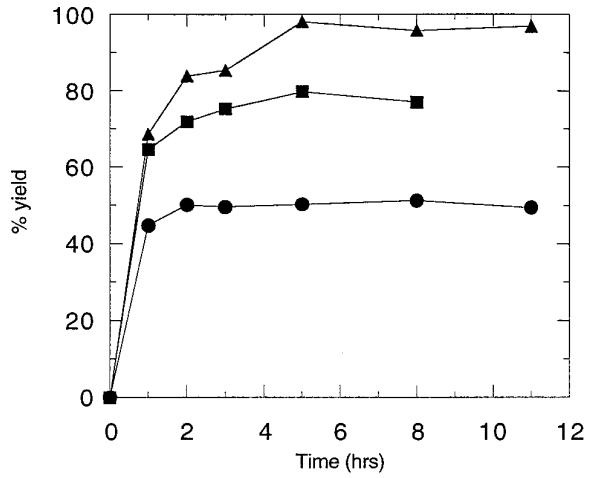

Figure 1. Effect of GdmCl on the kinetics of the oxidative renaturation of lysozyme. Renaturation was initiated by rapidly diluting reduced denatured lysozyme into renaturation buffer. Final conditions: 1 mg/mL lysozyme, 2 m*M* DTT, 5 m*M* GSSG, in 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 8, 25°C, and (●) 0.5 *M* GdmCl, (■) 0.75 *M* GdmCl, (▲) 1.25 *M* GdmCl.

8) and dissolved in 8 *M* GdmCl, 32 m*M* DTT, TE buffer. The amount of redissolved aggregated protein was determined by measuring absorbance at 280 nm (see above).

RESULTS

Optimization of Renaturation Conditions at 1 mg/mL

GdmCl Concentration Dependence of Renaturation

It is well known that chaotrophic agents such as urea and GdmCl denature or destabilize proteins. While high concentrations of GdmCl unfold the molten globule state, contrary to expectations, low concentrations were found to stabilize it (Hagihara et al., 1993). The presence of low concentrations of chaotrophic agents in the renaturation buffer may minimize aggregation and consequently improve yields. GdmCl employed during solubilization to unfold lysozyme may be used during the renaturation process to enhance refolding. Dilution of the solubilized protein directly into the renaturation buffer facilitates the refolding step by eliminating the need to remove the denaturant.

Systematic variation of the concentration of GdmCl during renaturation demonstrated that this parameter plays a significant role in determining renaturation rates and yields. Conditions can be found under which the amount of functional protein produced is drastically increased. Lysozyme at 1 mg/mL was renatured in buffer systems containing GdmCl levels ranging from 0.5 to 1.25 *M* (Fig. 1). Renaturation yields showed a marked increase as the concentration of GdmCl in the refolding solution increased. With 0.5 *M* GdmCl present, the absolute amount of renaturation is only about 50%, whereas over 95% of the lysozyme can be renatured in the presence of 1.25 *M* GdmCl. However, renaturation rates slowed in conjunction with the increase in GdmCl. At 0.5 *M* GdmCl, the maximum renaturation yield is achieved at about 1 h; while it takes 3 and 5 h to reach the maximum yield when the GdmCl concentrations are 0.75 and 1.25 *M*, respectively. Provided with sufficient time, protein folding in solutions containing greater quantities of GdmCl reached and surpassed renaturation yields attained at lower GdmCl concentrations.

The influence of GdmCl on the aggregation reaction can be monitored using light scattering techniques. The onset of aggregation was delayed and its extent reduced as GdmCl levels increased. In 0.5 *M* GdmCl, aggregation ensued immediately after dilution into the renaturation buffer (data not shown). But in 1.25 *M* GdmCl, there was no indication of turbidity until 30 min into renaturation.

Folding Additives in Nondenaturing Concentrations

Other solubilizing substances, such as L-arginine, can exert a similar beneficial effect to that observed when GdmCl is carried over from the denaturation solution (Rudolph, 1990). Addition of L-arginine to the renaturation buffer resulted in an increase in yield of active protein (Fig. 2). Lysozyme at 1 mg/mL was refolded in solutions containing 0.5 *M* GdmCl and from 0 to 1 *M* L-arginine. Increasing concentrations enhanced the yield but slowed the renaturation rate. The maximum

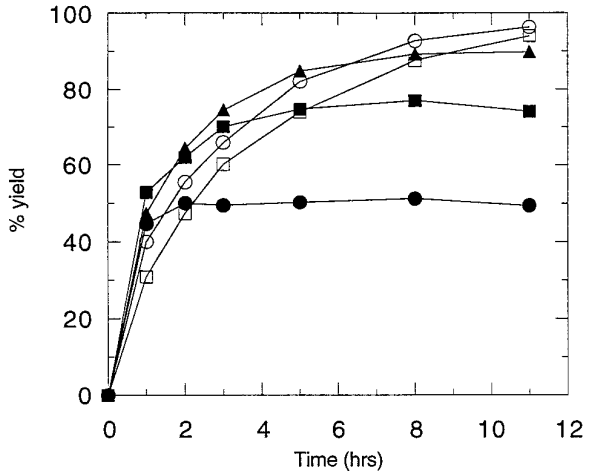

Figure 2. Effect of L-arginine on the kinetics of the oxidative renaturation of lysozyme. Renaturation was initiated by rapidly diluting reduced denatured lysozyme into renaturation buffer. Final conditions: 1 mg/mL lysozyme, 2 m*M* DTT, 5 m*M* GSSG, 0.5 *M* GdmCl, in 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 8, 25°C, and (●) 0 *M* L-arginine, (■) 0.25 *M* L-arginine, (▲) 0.5 *M* L-arginine, (○) 0.75 *M* L-arginine, and (□) 1 *M* L-arginine.

yield after 3 h of folding reached 75% in the presence of 0.5 *M* L-arginine, compared to only 50% in its absence. As expected, with time, yields continued to rise for solutions containing higher concentrations of L-arginine. After 11 h in 0.75 *M* L-arginine, 96% of the lysozyme regained activity, almost twice the activity recovered without this additive. The decrease in turbidity as the amount of L-arginine increased indicated that aggregation was reduced (data not shown).

GdmCl versus L-Arginine

Figure 2 confirms the previously demonstrated (Buchner and Rudolph, 1991; Buchner et al., 1992; Rudolph, 1990) effectiveness of L-arginine as a folding enhancer. However, although renaturation yields are greatly improved, renaturation times are also increased. A similar effect was observed when low concentrations of GdmCl were utilized as folding enhancers (Fig. 1). Figure 3 compares the effects of low concentration of GdmCl and L-arginine on the kinetics of oxidative renaturation of 1 mg/mL lysozyme. In the presence of 1.25 *M* GdmCl, 98% of lysozyme activity is recovered after 5 h, compared with only 50% activity recovery in the presence of 0.5 *M* GdmCl. Addition of 0.5 *M* L-arginine results in a 90% activity recovered after 8 h and if the amount of L-arginine added is increased to 1 *M*, 94% activity can be recovered in 11 h. Figure 3 shows that low concentrations of GdmCl are as effective as addition of L-arginine in increasing folding yields but they result in a much faster renaturation rate.

Thiol Concentration Dependence on Renaturation

The right mixture of low molecular weight thiol components in oxidized and reduced forms needs to be added to the renaturation buffer to allow disulfide bond formation and shuffling. Previous studies have indicated that optimum thiol concentrations in the renaturation buffer are 0.8–8 m*M* reduced glutathione (GSH) and 0.04–0.4 m*M* oxidized glutathione (GSSG) (Saxena and Wetlaufer, 1970). Jaenicke and Rudolph (1989) recommend 0.3 m*M* GSSG and 3 m*M* GSH or similar concentrations of other low molecular weight thiol reagents in oxidized and reduced form. GSH can be replaced with dithiothreitol (DTT) or dithioerythritol (DTE) (Rudolph, 1990), since oxidized glutathione is rapidly reduced by DTT or DTE to establish a GSH/GSSG reduction/oxidation system. At pH 8 and 25°C, the rate constant for the reduction of GSSG by DTT is 186 ± 16 min^{-1} M^{-1}, with an equilibrium constant of 229 ± 20 *M* (Rothwarf and Scheraga, 1992). A mixture of 2 m*M* DTT and 4 m*M* GSSG rapidly converts to 4 m*M* GSH, 2 m*M* GSSG, and 2 m*M* oxidized DTT (DTT_{ox}). Buchner and Rudolph (1991) found that the best DTE/GSSG mixture to properly fold Fab antibody fragments was 3 m*M* DTE and 4 m*M* GSSG for Fab concentrations of 0.060 mg/mL. The above thiol concentrations were optimized for oxidative renaturations at low protein concentrations (0.01–0.1 mg/mL) and might not be appropriate when folding a protein at 1 mg/mL or higher concentrations. A matrix approach was used in this study to determine the optimum concentrations of DTT and GSSG which give the highest yields of renatured lysozyme at 1 mg/mL when folded in the presence of 1 *M* GdmCl (Figs. 4a–d). DTT and GSSG concentrations were varied between 1 and 6 m*M* and 4 and 13 m*M*, respectively. Figure 4 shows that both yields and rates are strongly dependent on thiol concentrations in the renaturation buffer. Highest yields after 3 h of refolding were obtained at 2 m*M* DTT/4–7 m*M* GSSG and 4 m*M* DTT/7 m*M* GSSG. In addition, these conditions produced the lowest turbidities (data not shown). Figure 4 indicates that as DTT concentration increases, higher GSSG concentrations are needed to optimize yields, resulting in optimum ratios of GSH/GSSG between 0.8 and 3 (DTT/GSSG between 0.3 and 0.6). Figure 4 also shows that high total glutathione concentrations (13 m*M*) inhibit folding, even when the resulting GSH/GSSG ratios are within the optimum range (DTT concentrations of 4 and 6 m*M*). In the absence of oxidizing power (6 m*M* DTT, 4 m*M* GSSG) Figure 4 shows that no activity is recovered during the first 3 h, and in addition, no activity was observed after 24 h (data not shown), indicating that air oxidation plays a minimal role in these experiments.

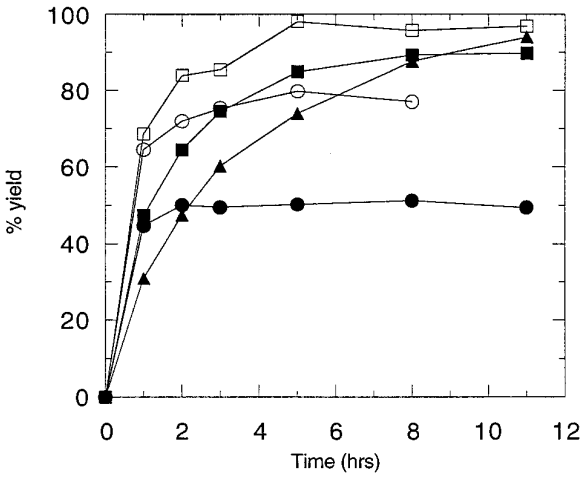

Figure 3. Effect of GdmCl and L-arginine on the kinetics of the oxidative renaturation of lysozyme. Renaturation was initiated by rapidly diluting reduced denatured lysozyme into renaturation buffer. Final conditions: 1 mg/mL lysozyme, 2 m*M* DTT, 5 m*M* GSSG, in 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 8, 25°C, and (●) 0.5 *M* GdmCl, (■) 0.5 *M* GdmCl, 0.5 *M* L-arginine, (▲) 0.5 *M* GdmCl, 1 *M* L-arginine, (○) 0.75 *M* GdmCl, and (□) 1.25 *M* GdmCl.

Renaturation at Higher Protein Concentrations (5 mg/mL)

Renaturing proteins in highly concentrated solutions would dramatically reduce the volumes of renaturation buffer that are needed to produce functional molecules. Because the aggregation reaction is higher order with

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