Efficient Refolding of Aggregation-prone Citrate Synthase by Polyol Osmolytes

HOW WELL ARE PROTEIN FOLDING AND STABILITY ASPECTS COUPLED?*

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Efficient refolding of proteins and prevention of their aggregation during folding are of vital importance in recombinant protein production and in finding cures for several diseases. We have used citrate synthase (CS) as a model to understand the mechanism of aggregation during refolding and its prevention using several known structure-stabilizing cosolvent additives of the polyol series. Interestingly, no parallel correlation between the folding effect and the general stabilizing effect exerted by polyols was observed. Although increasing concentrations of polyols increased protein stability in general, the refolding yields for CS decreased at higher polyol concentrations, with erythritol reducing the folding yields at all concentrations tested. Among the various polyols used, glycerol was the most effective in enhancing the CS refolding yield, and a complete recovery of enzymatic activity was obtained at 7 M glycerol and 10 µg/ml protein, a result superior to the action of the molecular chaperones GroEL and GroES in vitro. A good correlation between the refolding yields and the suppression of protein aggregation by glycerol was observed, with no aggregation detected at 7 M. The polyols prevented the aggregation of CS depending on the number of hydroxyl groups in them. Stopped-flow fluorescence kinetics experiments suggested that polyols, including glycerol, act very early in the refolding process, as no fast and slow phases were detectable. The results conclusively demonstrate that both the thermodynamic and kinetic aspects are critical in the folding process and that all structure-stabilizing molecules need not always help in productive folding to the native state. These findings are important for the rational design of small molecules for efficient refolding of various aggregation-prone proteins of commercial and medical relevance.

Aggregation of proteins during folding, both in vitro and in vivo, is known to lead to low native protein yields as well as the onset of several age-related diseases (1). Hence, there is a growing interest in developing strategies to prevent protein aggregation to enhance protein refolding yields and to design drugs for diseases involving protein aggregation. Several attempts have been made in this direction with successes as well as failures (2–4). In this study, we used citrate synthase (CS),1 a non-disulfide-bonded dimeric protein (~100 kDa) highly prone to aggregation during in vitro refolding (5–12), as a model protein. The aggregation-prone nature of CS during its refolding has made it an attractive model system to study the effect of molecular chaperones on the prevention of its aggregation and to develop strategies for enhancing protein refolding yields (8, 13–20). Using the molecular chaperones GroEL and GroES, up to 80% refolding of CS could be obtained at 10 µg/ml protein, whereas unassisted refolding was only 5% (7). Inspired by the GroEL/ES-assisted two-step folding mechanism, Rozema and Gellman (9) proposed an artificial chaperone-assisted refolding system in which a detergent was used to prevent aggregation, and cyclodextrin was added to remove the detergent, leading to the native structure formation. Using this strategy, a 65% refolding yield of CS was observed (21). Recently, a similar two-step strategy has also been applied using cycloamylose, and complete recovery of the refolded protein has been claimed (12). Despite its successful use in improving the refolding yield, the two-step artificial chaperone strategy is complicated, requiring the removal of the accessory agents before the enzyme can be effectively used.

Another useful strategy that has been applied to improve the refolding yield of proteins is to use small molecular mass additives in the refolding buffer. The advantage of using such a strategy over the artificial chaperone strategy is that it is convenient and cost-effective, and the additives need not be removed from the refolding buffers. One can also select compounds that are simple in structure and that are biocompatible, thereby offering a possibility to be used in vivo to correct protein misfolding and to prevent aggregation responsible for a growing number of human and animal diseases (22–24). Efforts are being made to enable the design of such compounds, referred to as “neutral crowders,” employing molecular dynamics simulations of protein associations in their presence (25). Low concentrations of denaturants such as arginine hydrochloride (2, 26, 27), polyethylene glycols (28–30), detergents (31, 32), and polyols and sugars (33–35) have been used to enhance the refolding yield of several proteins. Despite successful use of these molecules in enhancing protein refolding yields, their mechanism of action for the refolding process is far from clear. In this study, we used a series of polyol osmolytes with increasing numbers of hydroxyl groups in an attempt to enhance the refolding yield of CS as well as to investigate their mechanism of action given that their mechanism of action for enhancing protein stability is well understood (36–41). Because glycerol was found to be the most effective among the polyols used in enhancing the refolding yield, ex-

1 The abbreviations used are: CS, citrate synthase; GdmCl, guanidinium chloride.
tended studies were carried out with glycerol to understand its mechanism of action in detail. Understanding the mechanism of cosolvent-mediated folding of proteins and the prevention of their aggregation would be helpful not only in the rational design of additives for enhancing protein refolding yields, but also in the prevention of aggregation of proteins involved in several conformational diseases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Acetyl coenzyme A, CS (porcine heart), dithioerythritol, 5,5‘-dithiobis(2-nitrobenzoic acid), erythritol, oxalaceta, sorbitol, Tris, and xylitol were purchased from Sigma. Ethylene glycol (Qualigens India Ltd.), glycerol (Roth Chemicals), and guanidinium chloride (GdmCl; Sigma and ICN Biomedicals) were of the highest purity grade and were used as such. For protein concentration determination and enzyme assay, Hitachi U2900, Specord (Analytik Jena AG), and Cary spectrophotometers were used. For maintaining constant temperature, a Haake Technik GmbH water bath was used.

**Refolding Protocol**—The concentration of native CS was determined by measuring the absorbance at 280 nm using ε\textsubscript{280} = 1.78 for a 1 mg/ml solution in a 1-cm path length cuvette (42), and the enzyme was used without further purification. CS (1 mg/ml) was denatured in 0.1 M Tris, pH 8.1, containing 6.0 M GdmCl and 20 mM dithioerythritol for at least 1 h at room temperature (6).

Refolding experiments were carried out by diluting the denatured protein into the refolding buffer (0.1 M Tris-HCl, pH 8.1). In the case of cosolvent-assisted refolding, various cosolvents were added at different concentrations to the refolding buffer to which the denatured protein was added. Refolding was carried out by single shot dilution in a glass test tube, in which the denatured protein was diluted 1:100 in the refolding buffer by vigorous vortexing for 30–60 s. Extreme care had to be taken at this step because rapid mixing was essential for reproducibility and better refolding yields. Refolded samples were incubated at the desired temperatures until completion of the reaction. The refolding at 25 °C was complete in <2 h. CS activity assay was carried out to follow the formation of native CS molecules. CS catalyzes the following reaction: oxalacetate + acetyl coenzyme A → citrate + CoASH.

The free –SH groups of CoASH react with 5,5‘-dithiobis(2-nitrobenzoic acid) and form a mercapto ion, 2-nitro-5-thiobenzoate, which absorbs at 412 nm. The reaction mixture contained 100 mM Tris-HCl, 0.1 mM 5,5‘-dithiobis(2-nitrobenzoic acid), 0.047 mM acetyl coenzyme A, and 0.023 mM oxalaceta. For the activity assay, 5 or 10 μl of enzyme was added to make a solution with a final concentration of 1.0 ml, and the enzyme was recorded for 180 s. For calculating the change in absorbance, ε\textsubscript{280}/ε\textsubscript{412} = 280 and 338 nm with slit widths of 10 and 20 nm, respectively. The change in absorbance was recorded for 180 s. For calculating the change in absorbance (ΔA/Δt), the initial linear portion was used (43). All activity assays were carried out at 25 °C. The change in absorbance of the native protein, which was treated in the same way as the refolded sample but without denaturing, was taken as 100% while expressing the relative refolding yields. The enzyme assays were carried out at least in triplicate. The average values were reported, and the error in the activity measurements was not more than ±5%.

**Aggregation Kinetics**—Aggregation kinetics studies of CS were carried out by detecting light scattering of the aggregates formed during folding at 25 °C or after high temperature incubation at 45 °C using a Cary Varian Eclipse fluorometer and a 400-μl stopped fluorescence cuvette with constant stirring. Excitation and emission wavelengths were set to 500 nm with a slit width of 2.5 nm each. For folding studies, the mixing of the denatured protein was carried out manually in the spectrometer cuvette with stirring, yielding a final protein concentration of 10 μg/ml. For aggregation studies at 45 °C, 10 μg/ml CS was also used.

**Refolding Kinetics**—The refolding kinetics of CS in the absence and presence of polyls were monitored at 24 °C by fluorescence detection using a BioLogic SFM-300 stopped-flow module attached to a Jasco J-810 spectrophotometer with an addition photomultiplier tube for fluorescence detection. The excitation wavelength for intrinsic tryptophan fluorescence was 280 nm, and an interference filter with a peak transmittance at 340 nm was used for emission detection. A mixing ratio of 1/9 denatured protein/folding buffer (0.1 M Tris-HCl, pH 8.1) was used for the refolding of the protein in a 30-μl cuvette. Data were acquired up to 1 s for the detection of any fast phase with a time interval of 0.5–2 ms as well as up to 3 min with an interval of 0.1 s.

Measurements of refolding kinetics were carried out using an Applied Photophysics RX.2000 rapid kinetics setup based on a pneumatic ram drive system using a microvolume cuvette interfaced with a Varian Eclipse fluorometer. The mixing ratio for CS folding in this case was 1:10 using a 250-μl syringe for the protein solution and 2.5-ml syringe for the refolding buffer. The refolding protein concentration was 10 μg/ml. Experiments were carried out at 0.9, 2.7, 4.5, and 6.4 mM glycerol in 0.1 M Tris, pH 8.1, as well as in 1 mM each ethylene glycol, erythritol, xylitol, and sorbitol at 24 °C. The excitation and emission wavelengths were set to 338 nm with slit widths of 10 and 20 nm, respectively. The limitation of the instrumental setup allowed the first data point to be collected at 31 ms only for experiments with data collection up to 1 s. Both the fast phase (up to 1 s) and the slow phase (up to 3 min) were acquired. The 338-nm wavelength was selected based on equilibrium measurements of the fluorescence spectra of the native and denatured protein solutions. Furthermore, pH jump experiments to monitor the dimer formation kinetics were carried out by mixing, at a 1:1 ratio, a 100 μg/ml CS solution in 50 mM phosphate buffer, pH 5.8, with a 50 mM phosphate buffer solution, pH 8, yielding a final protein concentration of 10 μg/ml at pH 8.

### Results

**Effect of Polyols**—Table I shows the results of the effect of polyols, viz. ethylene glycol, glycerol, erythritol, xylitol, and sorbitol ranging from 0.5 to 9 mM, depending on their solubility, on the CS refolding yield at a concentration of 10 μg/ml and 25 °C. The polyols vary in the number of –OH groups in them: ethylene glycol has two, glycerol three, erythritol four, xylitol five, and sorbitol six. Except erythritol, which decreased the refolding yield at all concentrations, all other polyols led to an initial increase in the refolding yield, followed by a decrease at higher concentrations. Erythritol is known to increase the thermal stability of proteins (44), but resulted in a decrease in the refolding yields for CS. In contrast, ethylene glycol, which is known as a destabilizer of protein conformation (39), led to a gradual increase in the refolding yield of CS, with a maximum yield of 52% at 5 mM, whereas the control yield was 36%. There was a sharp decline in the refolding yield after 5 mM, with no refolding activity obtained at 9 mM.

Glycerol also led to a gradual increase in the refolding yield, and a very high refolding yield of 83% was obtained at 7 mM, followed by a decrease. It was not possible to use glycerol at concentrations higher than 9 mM, as there were mixing problems due to the high viscosity of the solutions. Glycerol has long been known to stabilize proteins against chemical and thermal denaturation. Gekko and Timasheff (36, 37) proposed that the solvophobic effect of glycerol is responsible for its stabilizing action. Glycerol has been used to increase the refolding yield of proteins (34, 45, 46). Glycerol has been used not only in in vitro studies, but also in in vivo studies, where it has been used to prevent the formation of the scrapie form (PrP\textsuperscript{Sc}) of the soluble prion protein (PrP\textsuperscript{Sc}) (22). The refolding yield obtained by us for CS in glycerol is comparable with that using the GroEL/ES system, in which a similar yield has been obtained (7). Both xylitol and sorbitol resulted in maximum refolding yields of 60

| Table I: Effect of polyols on the refolding yield of CS |
|-----------------------------------------------|
| Conc   | EG Glycerol | Erythritol | Xylitol | Sorbitol |
|--------|-------------|------------|---------|----------|
| 0.0 M  | 36          | 37         | %       |          |
| 0.5 M  | 11          | 37         | 34      | 35       |
| 1.0 M  | 35          | 51         | 7       | 60       |
| 1.5 M  | 11          | 54         | 38      |          |
| 2.0 M  | 49          | 56         | 8       | 17       |
| 3.0 M  | 46          | 67         |         |          |
| 5.0 M  | 52          | 71         |         |          |
| 7.0 M  | 32          | 83         |         |          |
| 9.0 M  | 0           | 71         |         |          |
inhibited the refolding of CS, but 1.5M urea and 7M glycerol led to a very high refolding yield. To inhibit the refolding completely, 3M urea was required in the presence of 7M glycerol.

Effect of Protein Concentration and Temperature—It has been reported previously that the refolding yield of CS depends on protein concentration (6, 7). As the protein concentration increased, there was a drastic decrease in the spontaneous refolding yield of the enzyme, with negligible activity observed at a refolding concentration of 50 μg/ml (Table II). The addition of 7M glycerol led to an increase in the refolding yield at 10 and 20 μg/ml almost to a similar extent but a smaller increase was observed relatively at 50 μg/ml. Refolding of proteins is also known to be highly temperature-dependent, with a tendency to form aggregates with increasing temperature, as a consequence of which, the refolding yield is reduced (47). This is quite obvious from the data on CS presented in Table III. The data on the effect of glycerol studied at various temperatures (10, 15, 25, 30, and 35 °C) reveal that glycerol helped to refold CS to a greater extent with increasing temperature, resulting in a 100% yield at 30 and 35 °C (Table III), at which the control activities were quite low. This result is much superior to the results obtained for CS refolding using molecular chaperones (7).

Addition of Glycerol at Different Time Intervals—To determine at which stage glycerol can affect the refolding of CS, glycerol was added at different time intervals during the folding of CS by manual mixing. The results show that there was almost a negligible effect of glycerol even as early as 15 s after refolding. No significant effect of glycerol on the CS refolding yield (activity) was observed when the addition was delayed further (data not shown). This suggests that the presence of glycerol, leading to an increase in the refolding yield, is necessary during the beginning or very early stages of refolding.

Compensation for the Urea Effect by Glycerol—Studies on the compensation effect of glycerol on the refolding of CS in the presence of various concentrations of urea were carried out. Urea was used as a cosolvent additive in the concentration range of 0.5–2M (Table IV). There was no effect of urea at 0.5M, whereas a further increase in its concentration led to a decrease in the refolding yield, with no refolding observed at 2M. It has been shown previously in the case of P22 tailspike protein folding that low concentrations of urea destabilize folding intermediates (48). It is possible that an intermediate in CS refolding may also be susceptible to low urea concentrations, which in turn decrease the refolding yield. To determine whether glycerol stabilizes folding intermediates and in turn increases the refolding yield, CS refolding was carried out at 7M glycerol in the presence of various concentrations of urea (Table IV). We observed that 1.5M urea almost completely inhibited the refolding of CS, but 1.5M urea and 7M glycerol led to a very high refolding yield. To inhibit the refolding completely, 3M urea was required in the presence of 7M glycerol.

Denaturation Transition and Thermal Inactivation—Denaturation transition curves measured by activity assay of CS (Fig. 1) in varying concentrations of GdmCl showed that CS was very sensitive to denaturation by GdmCl. At a concentration as low as 0.5M, it started losing activity rapidly. In the presence of 3M glycerol, however, the denaturation transition curve was shifted toward higher GdmCl concentrations. These experiments suggest that glycerol helps in the stabilization of the native CS structure against GdmCl denaturation. Glycerol concentrations higher than 3M were difficult to achieve due to mixing problems with GdmCl.

To study the effect of glycerol on the thermal inactivation of CS, inactivation kinetics experiments with the enzyme were performed at 45°C. This temperature was chosen because it has been reported that CS inactivates rapidly at this temperature, resulting in no activity after 20 min of incubation (18). Fig. 2 shows that there was a complete loss of enzymatic activity after incubation at 45°C for 25 min, whereas in the presence of 7M glycerol, 20% of the enzymatic activity was retained. This further suggests that glycerol stabilizes the native structure of CS against the effect of high temperature.

| Table II  | Effect of protein concentration on the refolding yield of CS in 7.0M glycerol at 25°C |
|-----------|----------------------------------|
| CS conc (μg/ml) | Spontaneous refolding | Glycerol-assisted refolding |
| 10         | 40 | 83 |
| 20         | 21 | 73 |
| 50         | 3  | 40 |

| Table III  | Effect of temperature on CS refolding in the absence and presence of 7M glycerol |
|------------|----------------------------------|
| Temperature (°C) | Spontaneous refolding | Glycerol-assisted refolding |
| 10         | 47 | 51 |
| 15         | 50 | 47 |
| 25         | 40 | 83 |
| 30         | 29 | 100 |
| 35         | 11 | 100 |

| Table IV  | Effect of urea (Part A) and a mixture of urea and glycerol (Part B) on CS refolding yield at 25°C |
|-----------|----------------------------------|
| Refolding yield (%) |
| Part A, Urea |
| 0.0M | 35 |
| 0.5M | 38 |
| 1.0M | 26 |
| 1.5M | 4  |
| 2.0M | 0  |
| Part B, Solvent |
| Spontaneous | 33 |
| 1.5M urea + 7.0M glycerol | 68 |
| 2.5M urea + 7.0M glycerol | 6  |
| 3.0M urea + 7.0M glycerol | 0  |
Refolding and Aggregation Kinetics—Fig. 3 presents data on the refolding kinetics in the absence and presence of 7 M glycerol determined by activity measurements. The addition of glycerol slowed down the kinetics of refolding as evidenced by the rate constants of 0.213 and 0.126 min⁻¹ in 7 M glycerol compared with 0.635 and 0.148 min⁻¹ in the control buffer at 10 and 35 °C, respectively. Data on the aggregation kinetics of CS during refolding by manual mixing in the presence and absence of 1 M polyols as monitored by fluorescence at 25 °C are shown in Fig. 4a. Polyols suppressed the aggregation of CS depending on the number of hydroxyl groups in them, which is in accordance with their effect on the thermal stability of proteins (39, 44). Interestingly, erythritol, which led to a decrease in the refolding yield, resulted in suppression of aggregation, suggesting that factors other than aggregation of CS also contribute to the polyol-mediated refolding of the protein.

Because glycerol proved to be the most effective agent for refolding, aggregation kinetics studies were carried out in the absence and presence of 1–9 M glycerol. We observed that aggregation decreased with the increase in glycerol concentration and was nearly abolished at 7 and 9 M (Fig. 4b). These results suggest a good correlation between the ability of glycerol to enhance the CS refolding yield and to suppress protein aggregation during folding. To investigate further the role of glycerol in stabilizing CS conformation, the effect of increasing glycerol concentrations on CS aggregation kinetics was determined at 45 °C (Fig. 4c). Again, we observed that glycerol prevented the aggregation of CS when incubated at elevated temperatures, suggesting its structural stabilizing effect on CS, in line with the general stabilizing effect of glycerol on several proteins.

Fast Kinetics Measurements—Because CS is highly aggregation-prone during folding in buffer even at a low concentration of 10 μg/ml, it is likely that the conformational changes during folding would be obscured upon spectroscopic detection. However, because aggregation was suppressed during folding in the presence of polyols, especially at high concentrations of glycerol, it was worthwhile to investigate any effect of polyols on the folding kinetics of the enzyme. To achieve this, stopped-flow kinetics measurements were carried out using a BioLogic stopped-flow system and an Applied Photophysics rapid kinetics accessory attached to a fluorometer. It was not possible to detect folding by circular dichroism due to the high concentrations required for detection, and hence, fluorescence detection was preferred.

The fluorescence emission spectra of native, denatured, and refolded aggregated CS in buffer and in 7 M glycerol at 10 μg/ml are shown in Fig. 5. There was a red shift of the emission maximum from 338 nm for the native protein to 352 nm for the denatured protein. The refolded sample in buffer containing aggregated species did not show any shift in the emission maximum but showed a reduced intensity, and the same was true for the refolded sample in 7 M glycerol. The considerable difference in the fluorescence intensities between the native and denatured proteins at 338 nm formed the basis of the rapid kinetics experiments.

The refolding kinetics measurements for CS in the folding buffer monitored by the BioLogic stopped-flow module/Jasco setup showed erratic behavior, which was likely due to protein aggregation (data not shown). However, the refolding kinetics in 1 M polyols and increasing concentrations of glycerol showed a slow decay of fluorescence with time. We observed that even the native control mixed simply with the folding buffer showed a decay in fluorescence, although to a lesser extent. This could be due to photo-oxidation effects coupled with mixing effects in
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DISCUSSION

Effect of Polyols on the CS Refolding Yield—The data in Table I reveal that different polyols refolded CS to varying extents and that, in all cases, there was a decrease in the refolding yield at high polyol concentrations, except for erythritol, which resulted in a decrease at all concentrations. This is in stark contrast to the results obtained for the stability of proteins monitored by their $T_m$ values in polyols. Polyols are known to increase the thermal stability of a wide variety of proteins (36–41, 50). The thermal stability of proteins is enhanced by several degrees in temperature depending on their concentration, and in some cases, increases in the $T_m$ are as high as $15^\circ$C at 2–3 m (41, 44). These compounds are also known to retain the enzymatic activity of proteins upon incubation at higher temperatures (51–53). It has been shown that the greater the increase in the $T_m$ of a protein in the presence of a polyol, the greater the activity retained in its presence, and that the longer the carbon chain length of a polyol or the number of hydroxyl groups in it, the better the thermal stabi-
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The stabilization provided by polyols could not be carried out due to the highly aggregation-prone nature of CS. Based on preferential interaction studies of proteins carried out in the presence of sorbitol in the native and denatured states, it has been proposed that polyols are likely to favor the native state over the denatured state (54), suggesting further that these compounds may also be helpful in the refolding of proteins from their denatured state to a more compact native state. Recent studies carried out by Bolen and Baskakov (55) demonstrate that the addition of certain osmolytes results in the compaction of RNase A. Compaction of α-synuclein, a natively unfolded protein, and acid-denatured cytochrome c has been observed in the presence of glucose as well as dextran, which are protein structure stabilizers (56, 57). The structure-stabilizing polyols are also expected to lead to such an effect and in turn favor the compact folded state.

Sorbitol has been shown to increase the $T_m$ of a variety of globular proteins (41). It is known to be preferentially excluded from the protein surface and has a weak affinity for polar groups on the protein surface (38, 39). Increases in the $T_m$ of globular proteins by sorbitol are due to their preferential hydration, which is a consequence of the increase in the surface tension of solvent water (41). Polyols, including sorbitol, have been shown to stabilize the acid-unfolded molten globule state of cytochrome c by strengthening hydrophobic interactions that overcome electrostatic repulsion at low pH between charged residues (58). In a recent study on the nucleocapsid protein of rhadovirus, sorbitol has been shown to reduce aggregation after expression of the protein in Escherichia coli. It has been shown that sorbitol can decrease the internal motions and thus favor the more compact state of the protein by fluorescence anisotropy (59). Sorbitol has been shown to increase the refolding yield of CS at 1.0 m to a maximum extent, and the refolding is dependent on sorbitol concentration (Table I). Because sorbitol is known to be preferentially excluded from the protein surface even in its denatured form, it is less likely that it may interact with the denatured or an intermediate state during folding. Hence, it is likely to exert its effect on folding by altering the structure and properties of water around the folding protein molecule. Sorbitol is known to increase both the surface tension of water (41) and its viscosity. It is therefore likely that it may critically control the folding kinetics of CS depending on its concentration, in addition to its general stabilizing effect based on thermodynamic considerations.

The stabilizing effect of xylitol has been shown to be less than that of sorbitol (41). However, its effect on CS refolding is almost similar to that of sorbitol. Like sorbitol, xylitol has also been shown to stabilize the molten globule state of cytochrome c (58). A higher concentration of xylitol (2 M) led to an appreciable decrease in the refolding yield of CS, which again points to factors like the solvent viscosity being responsible for affecting the subunit folding kinetics and in turn preventing protein-protein association. Unexpectedly low refolding yields obtained in the presence of erythritol at all concentrations studied (0.5–2.0 M) suggest an anomalous behavior of erythritol given that erythritol is also a protein structure stabilizer, although it stabilizes proteins to lesser extents compared with sorbitol (39). The data on CS aggregation kinetics during refolding in erythritol and other polyols shown in Fig. 4a also follow the usual trend for the ability of the polyols to stabilize proteins depending on the number of hydroxyl groups in them. The substantial reduction in the CS refolding yield in erythritol could arise from either the misfolding of monomeric CS in its presence or its ability to prevent the subunit association that leads to the native dimer. Further studies on other aggregation-prone proteins in polyols will be required to clarify the anomalous effect of erythritol.

Glycerol, which is a mild stabilizer of protein conformation compared with the other polyols studied, led to a very high recovery of refolding yield, although at a much higher concentration of 7 M (Table I). However, at a concentration of 1 M, its effect on refolding was similar to that of sorbitol and xylitol. It is interesting to note that, of these three polyols, sorbitol increases the $T_m$ of proteins to the largest extent, followed by xylitol and glycerol (39, 44). This further suggests that, in the polyol-mediated folding of CS, factors other than thermodynamics contribute as well. Glycerol has long been known as a protein stabilizer, and its stabilization mechanism has been proposed by Gekko and Timasheff (36, 37) and by Timasheff (60). Glycerol leads to the enhancement of hydrophobic interactions as a consequence of an increase in the solvent ordering around proteins. Glycerol is unusual in its properties in that it decreases the surface tension of water but increases its viscosity, unlike other polyols. Preferential hydration of proteins by glycerol is considered to be due to its solvophobic effect (36, 37). It has been shown that glycerol affects the hydrogen exchange rates of protons in myoglobin, leading to slow exchange rates, as a result of which, the dynamic motion of the folded core of the protein is dampened (61). The magnitude of the increase in the refolding yield of CS in 7 M glycerol is similar to that observed in the presence of the molecular chaperones GroEL and GroES (7). The higher refolding yield of CS in glycerol may be due to the stabilization of its folding intermediates. Even though increasing glycerol concentrations are known to increase the stability of proteins at all concentrations (37, 62), the decrease in refolding at high concentrations of glycerol could be due to its ability to considerably slow down the kinetics of refolding at very high concentrations, which could result in off-pathway aggregation. An optimum glycerol concentration seems to be essential in helping strike a balance between its stabilizing effect governed by thermodynamic principles and its effect on the kinetics of folding. A linear correlation between the thermal stability and folding kinetics as a function of pH has been observed using lysozyme as a model (63). However, our results suggest that osmolyte addition could critically alter this balance depending on the nature and the concentration of the molecule used.

Ethylene glycol is known to be a mild destabilizer of proteins (39), as is evident from its ability to decrease the $T_m$ of proteins. However, it has been observed to slowly increase the refolding yield of CS up to 5 M, after which it decreases drastically, with no activity observed at 9.0 M. Its destabilizing effect on proteins is suggested to be a result of the weak interaction of its methylene groups with the non-polar regions of the partially unfolded protein as the temperature is increased. It may thus help prevent CS aggregation by its weak binding to such exposed groups during refolding, even though it may have a slight destabilizing effect on protein conformation. Moreover, the destabilizing effect observed in terms of a decrease in the $T_m$ of proteins occurs at elevated temperatures close to the transition temperatures. Because CS refolding is carried out at room temperature, it is less likely to affect its stability. In addition, we have observed that the addition of ethylene glycol does not affect the activity of CS. Low concentrations of denaturing agents such as urea, GdmCl, and arginine hydrochloride have been successfully employed in the folding of proteins (27, 64). It is quite likely that ethylene glycol may work in a similar manner to these agents by its ability to weakly interact with the exposed hydrophobic groups and thus help prevent aggregation.

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2 A. Tiwari and R. Bhat, unpublished data.
Results on CS refolding in the presence of various polyols suggest that, even though polyols suppress aggregation of CS in a systematic manner and are known to linearly increase the thermal stability of proteins depending on the number of hydroxyl groups in them, similar systematic effects have not been observed in the case of protein folding, even though considerable increases in the refolding yields have been attained. The likely explanation for these non-parallel effects is that an increase in the $T_m$ of proteins by the polyol osmolytes involves the unfolding of the native protein wherein the cosolvent interacts either directly or indirectly only with the protein surface, whereas as the effect of these osmolytes during refolding involves the unfolded peptide chain having dynamic conformation, the folding of which in a cosolvent would involve kinetic aspects of folding as well. A balance between the preferential exclusion of these polyols from the native protein surface and the ability of some of them to weakly interact with the unfolded and the folding polypeptide chains should govern their effect on the protein folding process.

Measurements of refolding kinetics using fluorescence as a probe were unable to detect either a fast or slow folding event in the presence of 1 mM polyols as well as increasing concentrations of glycerol up to 6.4 M. The data again suggest that the refolding kinetics of CS involving both the subunit folding as well as the monomer association to form the native dimer are a very fast process. The presence of polyols is therefore required either in the beginning or in the very early stages of folding. pH jump studies on dimer formation also indicate that dimer formation is a very fast process and occurs in a <30-ms time interval, which was beyond the detection time of the rapid kinetics device used. However, the process of dimer formation by pH jump cannot be considered to be the same as dimer formation during refolding from the 6 M GdmCl-denatured protein, which involves the initial removal of the denaturant, leading to monomer folding, followed by dimer association to yield the native state.

Mechanistic Analysis of the Effect of Glycerol on the Refolding Process—CS is highly susceptible to aggregation, and its propensity to form aggregates during refolding increases with increasing protein concentrations (6). At higher concentrations, folding becomes rate-limiting, and aggregation increasingly competes with correct association of the subunits. The reason for this “kinetic partitioning” is that the recognition of a specific interface within a given assembly structure requires complementarity between the subunits. Thus, folding of the monomers needs to have reached a stage close to the native conformation before correct docking can occur. If collision takes place in advance, at a stage when folding intermediates still expose the wrong contact sites, it is likely to lead to aggregation (65). In this study, increases in the protein concentration led to a decrease in the refolding yield, and this phenomenon was observed for the unassisted as well as for the glycerol-assisted refolding (Table II).

To explain the behavior of glycerol at different temperatures, spontaneous refolding and glycerol-assisted refolding were carried out at different temperatures. Temperature has a complex effect in the case of glycerol-assisted refolding because, at lower temperatures (10 and 15 °C), it did not increase the refolding yield at all (Table III). This may be due to very high viscosity of glycerol solutions at 10 and 15 °C, as is evident from the reactivation kinetics (Fig. 3, a and b), which glycerol slowed down considerably. The most striking effect of glycerol was observed at 30 and 35 °C, at which almost complete recovery of the protein activity was obtained. At these temperatures, glycerol not only increased the refolding yield, but also stabilized native CS against time-dependent inactivation (data not shown). A combination of these effects should be responsible for 100% recovery of enzymatic activity. The stabilizing effect of glycerol on CS was also evident in the thermal inactivation experiment, in which the addition of 7 M glycerol resulted in up to 20% protection of enzymatic activity after incubation for 25 min at 45 °C (Fig. 2). Also, glycerol suppressed the aggregation of CS in a concentration-dependent manner upon incubation at 45 °C (Fig. 4c). Together, these results suggest why glycerol is more effective in increasing the refolding yield at higher temperatures compared with lower temperatures.

The data in Tables II and III clearly show that glycerol helps refold CS even under non-permissive conditions. Glycerol is required either from the beginning or in the very early stages of refolding, and once the committed step is passed, there is no effect of glycerol anymore. This result also indicates that the aggregation phenomenon takes place very rapidly in CS and that the intermolecular interactions due to the exposed hydrophobic residues take place at very early stages during folding. It further indicates that, once the aggregates are formed, glycerol is not able to reconvert the aggregates to the native state. A similar action of glycerol has also been observed in the case of folding of rhodanese (66). Experiments carried out on the refolding of CS in the presence of the molecular chaperones GroEL and GroES also suggest that they cannot reconvert the aggregates to the native CS molecule (6). A decrease in aggregation with increasing glycerol concentrations during refolding from the denatured state (Fig. 4b), which almost parallels the effect on the refolding yields observed in glycerol (Table I), suggests that suppression of aggregation by glycerol is a prime factor in glycerol-mediated refolding of CS to the native state. A decrease in refolding at 9 M glycerol could be attributed to solution viscosity being so high as to slow down the refolding kinetics drastically and lead to off-pathway aggregation, which is evident from the reduced magnitudes of the rate constants observed for CS refolding in 7 M glycerol (Fig. 3). Recently, Russo et al. (67) studied the effect of some osmolytes, including glycerol, on the folding kinetics of human immunophilin C22A mutant FKBP12, in which they observed enhanced folding rates in the presence of 1 mM glycerol and other osmolytes and concluded that the osmophobic effect governing protein stability is applicable to protein folding as well. In other words, the increased folding rate in the presence of osmolytes could be explained by their ability to stabilize proteins. However, another recent report determining the folding rates of chymotrypsin inhibitor-2 in the presence of the viscosgens ethylene glycol and polyethylene glycol points out that there is an increase in the folding rate at lower concentrations of the viscosgens, followed by a decrease at higher concentrations (68). These observations, along with our concentration-dependent studies on glycerol as well as other polyols, clearly point out that the folding kinetics of CS could be critically affected by bulk solution properties such as viscosity.

The addition of urea reduced the refolding yield in a concentration-dependent urea-assisted refolding experiment, and no refolding was observed at 2.0 M urea (Table IV, Part A). It may be possible that low concentrations of urea destabilize the essential folding intermediates and in turn promote the aggregation process, which leads to a decrease in the refolding yield. Low concentrations of urea and arginine hydrochloride have been found to increase the refolding yield of several proteins (27, 64, 69, 70). It has been suggested that, even though low concentrations of urea may slightly destabilize the native structure, its addition helps prevent aggregation by mildly solubilizing the intermediate aggregation-prone states, resulting in higher refolding yields. In the case of CS, it is known that its conformation is very sensitive to even low concentrations of
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GdmCl (5). Hence, the destabilizing effect in the presence of urea is expected to be much more pronounced than for other proteins for which refolding yields have increased. A similar action of urea was also observed in the case of the P22 tailspike protein (48).

The denaturation transition curve (using activity as a tool) revealed that the transition was shifted to higher GdmCl concentrations in the presence of glycerol (Table 1), indicating that glycerol stabilizes the native CS structure. Glycerol-induced stabilization of intermediates has also been observed in l-amino acid oxidase, in which glycerol is known to favor the hydrophobic network of partially folded structures and in turn stabilize the intermediates and favor the more compact state (71, 72). Therefore, this suggests that glycerol may help form certain folding intermediates that are stabilized by hydrophobic interactions and help prevent intermolecular protein association. However, no such intermediates were detectable in the rapid kinetics experiments. The concentration dependence of the folding of CS in the presence of glycerol further suggests that the bulk solution properties of glycerol, including solvent viscosity, may also play a critical role in preventing intermolecular protein association, and an interplay of these properties of glycerol is critical for obtaining optimum refolding yields.

In conclusion, polyol osmolytes, except erythritol, help refold of CS considerably, with glycerol leading to the complete recovery of enzymatic activity upon folding. There is a good correlation between the increase in the refolding yield in the presence of glycerol and its ability to suppress protein aggregation. The decrease in the refolding yield at high polyol concentrations suggests that, in addition to the thermodynamic considerations, kinetic factors during refolding in the cosolvents are important as well. The possibility of weak interactions of some of the polyols with the unfolded or the folding peptide chain and the extent of exclusion from the native protein molecule could further critically affect the folding process. A balance of these factors leads to productive folding or off-pathway aggregation of CS. The findings suggest that small structure-stabilizing organic molecules could be used as effective agents in preventing protein aggregation and in the therapy of several aggregation-related debilitating diseases.

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