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Renaturation of citrate synthase: Influence of denaturant and folding assistants

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Abstract

Citrate synthase (CS), which has been denatured in either guanidine hydrochloride (GdnHCl) or urea can be assisted in its renaturation in a variety of ways. The addition of each of the assistants—bovine serum albumin (BSA), oxaloacetate (OAA), and glycerol—promotes renaturation. In combination, the effect of these substances is additive with respect to the yield of folded CS. The report of Buchner et al. (Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F.X., & Kiefhaber, T., 1991, *Biochemistry 30*, 1586-1591) that refolding of CS is facilitated by the GroE system (an *Escherichia coli* chaperonin [cpn] that is composed of GroEL [cpn60] and GroES [cpn10]) has been confirmed. However, we observed substantially higher yield of reactivated CS, 82%, and almost no reactivation in the absence of GroES, <5%, whereas Buchner et al. reported 28% and 16%, respectively. In addition, we find that GroE-assisted refolding is more efficient for CS denatured in GdnHCl than for CS denatured in urea. This result is discussed in light of the known difference in the denatured states generated in GdnHCl and urea. Because GroEL inhibits the BSA/glycerol/OAA-assisted refolding, this system will be useful in future studies on the mechanism of GroE-facilitated refolding.

Keywords: citrate synthase; denaturation; protein folding; renaturation

It is accepted and adequately demonstrated (see Ghèlis & Yon, 1982, for review) that most denatured proteins, monomeric or polymeric, are renatured if proper conditions are determined. Generally speaking, one has to have low protein concentration during renaturation to minimize undesirable side reactions, such as aggregation and sulfhydryl oxidation. Also, the addition of ligands, such as substrates of enzymes, during renaturation often increases the rate and extent of renaturation. In one case the addition of low concentrations of detergents increased the degree of renaturation of a protein (Tandon & Horowitz, 1987; Mendoza et al., 1991b).

In eukaryotes, citrate synthase (CS) is encoded in the nucleus and transported into the mitochondrial matrix. Because it appears that such proteins are transported as nonnative structure polypeptides (Baker & Schatz, 1991), it is of interest and important to know the propensity of denatured CS to refold into its native conformation. Recent studies have indicated that proteins known as chaperonins (cpns) are involved in the correct folding of transported mitochondrial proteins (Ostermann et al., 1989; Kang et al., 1990).

The denaturation and renaturation of CS has been the subject of several studies (Greenblatt & Sarkissian, 1972; West et al., 1990; Buchner et al., 1991). The denaturation of CS has been studied by Wu and Yang (1970) and by Srere (1966). Two of the renaturation studies stand in stark contrast to each other in that one defines conditions for renaturation of CS after guanidine hydrochloride (GdnHCl) unfolding (Greenblatt & Sarkissian, 1972), whereas the other claims that no renaturation occurs (West et al., 1990). A recent communication has shown that the GroE system can effect the renaturation of GdnHCl-denatured CS (Buchner et al., 1991). The GroE system consists of a pair of cpns, GroEL (cpn60) and GroES (cpn10), from *Escherichia coli*, that have been shown to

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be effective in the refolding of denatured Rubisco (ribulose bisphosphate carboxylase: Goloubinoff et al., 1989) and a number of other proteins (Martin et al., 1991; Mendoza et al., 1991a).

We have, therefore, reinvestigated the renaturation of urea- and GdnHCl-denatured pig CS and defined conditions that allow for the renaturation and recovery of up to 50% of the total activity without the aid of cpns. We have also extended the studies on the renaturation of CS with the GroE system. Here, we report much higher recoveries of CS activity than previous studies, and we show that GroE-assisted refolding is exquisitely dependent on GroES in addition to ATP. The latter finding is particularly important for evaluating CS as a protein folding system that will be used to study the role of GroES in the mechanism of GroE function.

Results

Effect of dilution on CS activity

The renaturation technique used involves the dilution of the denaturant to a low concentration, and consequently the protein (CS) also undergoes a similar dilution. Our past experience with CS showed that its dilution to low protein concentrations is accompanied by a loss of its activity. Dilution to final concentrations of 10 μg/mL or less causes a 30% (in glass) or 50% (in plastic tubes) immediate loss of activity (data not shown). The dilution inactivation is prevented by the addition of bovine serum albumin (BSA) to the diluent. This observation (not specifically noted by others) will affect the apparent recovery of enzyme activity depending upon the control used in the experiment. In this paper we reference recovery with the activity of undiluted stock CS, assuming no losses because most diluents contain BSA (see below).

Repeat of previous experiments on CS renaturation

It was reported for CS denatured in GdnHCl that (1) CS renaturation occurs after dilution into buffer containing high concentrations of KCl (Greenblatt & Sarkissian, 1972), and (2) no CS renaturation occurs (West et al., 1990). We repeated these experiments as exactly as possible. Low recovery (5%) of activity was obtained using the conditions of Greenblatt and Sarkissian (1972), and no recovery was obtained with the conditions of West et al. (1990). The difference in the two experiments is that West et al. (1990) did not include dithiothreitol (DTT) in their denaturing solution.

Non-GroE-assisted renaturation

Because we knew that BSA protects against dilution inactivation and oxaloacetic acid (OAA) protects CS against urea and thermal denaturation (Srere, 1966), we tried these protectants as possible aids in renaturation. Glycerol has also been used to store enzymes for long periods of time, so this compound was also tried in renaturation experiments. CS has buried SH groups that could be oxidized on denaturation, so the effect of DTT in the denaturation and reactivation incubations was tested.

CS activity assays performed immediately after 8 M urea or 6 M GdnHCl treatment show that all CS activity was lost. Following a 2-h incubation in renaturation solution, there was a small (1–4%) but measurable activity restoration (Table 1). If OAA was added to the diluent, then there was about 10% recovery of enzyme activity. If the diluent contained BSA, then 25–35% of the activity was recovered. The dilution of denatured enzyme into a buffer containing both BSA and OAA gave a recovery of about 40% of the original activity. The addition of glycerol also assists renaturation, and the effect of the three substances is additive. Under most renaturation conditions, the yield is slightly greater (by 5–10%) for CS denatured in GdnHCl compared to CS denatured in urea.

The presence of DTT in the refolding buffer had only a small effect on renaturation. However, DTT in the urea denaturation buffer did result in higher recovery of activity upon renaturation. Furthermore, if DTT was omit-

Table 1. *Effects of BSA, OAA, and glycerol and the GroE system on renaturation of urea-denatured and GdnHCl-denatured pig citrate synthase*

	Recovery (%)	
Additions to refolding buffer	From urea[a]	From GdnHCl[b]
None	3	4
OAA	15	11
BSA	26	34
Glycerol 25%	28	17
BSA + OAA	45	42
OAA + glycerol 25%	27	23
BSA + glycerol 25%	40	62
BSA + glycerol 25% + OAA	49	65
GroEL + GroES + ATP + K^+ + Mg^{2+}[c]	42	82

[a] The enzyme was denatured by 8 M urea in 0.1 M Tris/HCl buffer, pH 8.1, containing 3 mM DTT. The enzyme concentration is 4 μM, and the incubation time is 30 min at room temperature. Renaturation was done by 20-fold dilution into 0.1 M Tris/HCl buffer, pH 8.1, containing the additions of 0.5 mM OAA, 0.4 mg/mL BSA, and 25% glycerol as indicated. Assay for the recovery of the activity was made after 2 h incubation at room temperature.

[b] The enzyme was denatured by 6 M GdnHCl in 0.1 M Tris/HCl buffer, pH 8.0, containing 20 mM DTT. The concentration of the enzyme was 10 μM, and the incubation time was 60 min at room temperature. Renaturation was done by 100-fold dilution into 0.1 M Tris/HCl buffer, pH 8.1, containing the additions of 0.5 mM OAA, 0.4 mg/mL BSA, and 25% glycerol as indicated. Assay for the recovery of the activity was made after 2 h incubation at room temperature.

[c] These data were taken from Figure 5. See Figure 5 for conditions.

ted from the GdnHCl denaturing solution, no recovery of activity was obtained upon dilution into a renaturing solution containing BSA and OAA.

Renaturation was essentially complete at 40 min in refolding buffer containing BSA and OAA. No essential difference was seen between urea- and GdnHCl-denatured enzyme during this time period (Fig. 1). If BSA or glycerol was initially omitted and then added 10 min after dilution into refolding buffer, there was little or no recovery of activity.

The recovery of activity was dependent upon the concentration of BSA in the refolding buffer, and the response was similar for CS denatured in either urea or GdnHCl (Fig. 2). For a concentration of CS monomers equal to 0.2 μM, the effect of BSA was saturated at 6 μM (0.4 mg/mL), or 30 BSA molecules for every CS monomer. However, it can be seen that only 3 BSA molecules (0.6 μM) per CS monomer is about half as effective in assisting folding as the saturating BSA concentration. Therefore, near-stoichiometric amounts of BSA support significant levels of folding.

For CS denatured in either urea or GdnHCl, a broad pH optimum was observed between 7.0 and 8.5 for renaturation in $KHPO_4$ buffer with BSA (Fig. 3).

As expected, increasing the CS monomer concentration in the refolding buffer led to a decrease in recovery of CS activity. Highest recovery was seen with 0.1 μM CS monomers (Fig. 4).

In addition to BSA, several other proteins were tested as folding assistants. Fumarase was ineffective, whereas mitochondrial malate dehydrogenase and lysozyme were much less effective than BSA. The lipid content of BSA (0 or 6 mol fatty acid/mol) was not related to the level of renaturation.

Refolding did not occur at 0 °C in the presence of BSA and OAA, but when the temperature was raised to room temperature, refolding proceeded normally. The same level of refolded CS was obtained at 37 °C as was obtained at room temperature.

It has been reported that detergents have a positive ef-

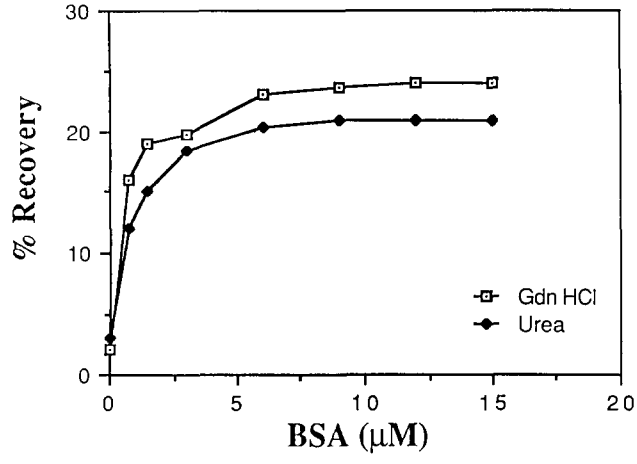


Fig. 2. Effect of BSA concentration on renaturation. Denaturation: CS was denatured either by 6 M GdnHCl or by 8 M urea in 0.02 M $KHPO_4$ buffer, pH 7.5, containing 3 mM DTT and 2 mM EDTA. The concentration of CS was 4 μM (monomer), and the incubation time was 30 min at room temperature. Renaturation: The denatured CS was diluted 20-fold in 0.02 M $KHPO_4$ buffer, pH 7.5, containing 0–15 μM BSA. Assay for recovery of the activity was made after 2 h incubation at room temperature.

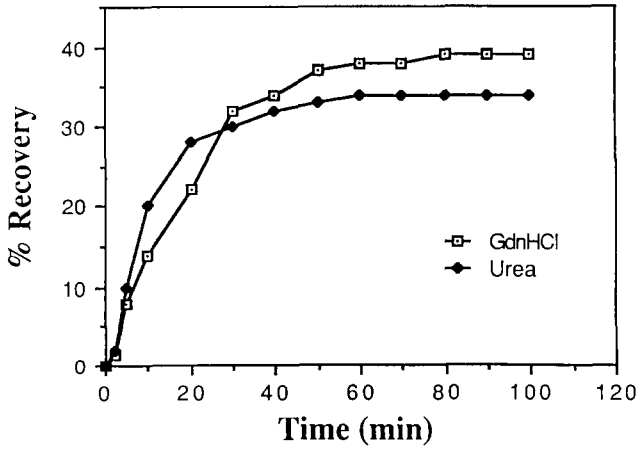


Fig. 1. Time course of renaturation in BSA and OAA. Denaturation: CS was denatured either by 6 M GdnHCl or by 8 M urea in 0.02 M $KHPO_4$ buffer, pH 7.5, containing 3 mM DTT and 2 mM EDTA. The concentration of CS was 4 μM (monomer), and the incubation time was 30 min at room temperature. Renaturation: The denatured CS was diluted by 20-fold in 0.02 M $KHPO_4$ buffer, pH 7.5, containing 6 μM BSA and 0.5 mM OAA. Assay for recovery of the activity was made after the indicated incubation time at room temperature.

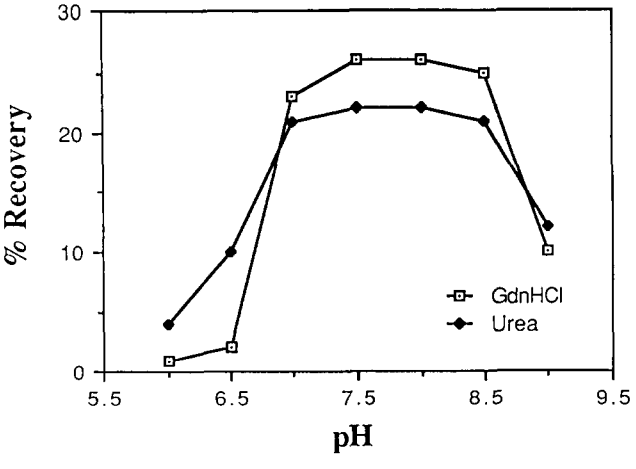


Fig. 3. Effect of pH on renaturation. Denaturation: CS was denatured either by 6 M GdnHCl or by 8 M urea in 0.02 M $KHPO_4$ buffer, pH 7.5, containing 3 mM DTT and 2 mM EDTA. The concentration of CS was 4 μM (monomer), and the incubation time was 30 min at room temperature. Renaturation: The denatured CS was diluted by 20-fold in 0.02 M $KHPO_4$ buffer with indicated pH containing 6 μM BSA. Assay for recovery of the activity was made after 2 h incubation at room temperature.

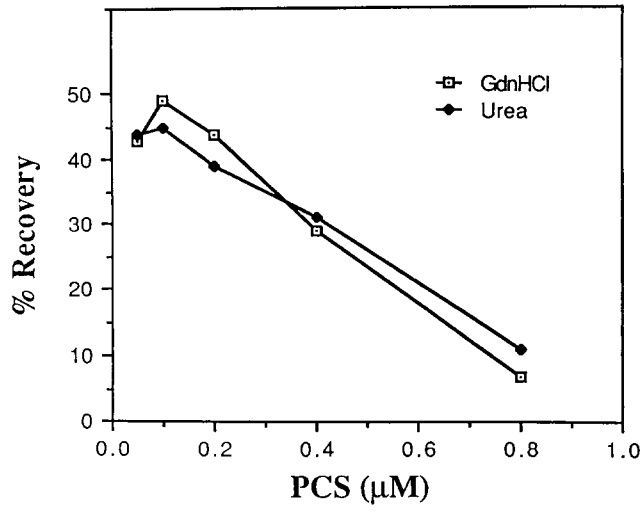
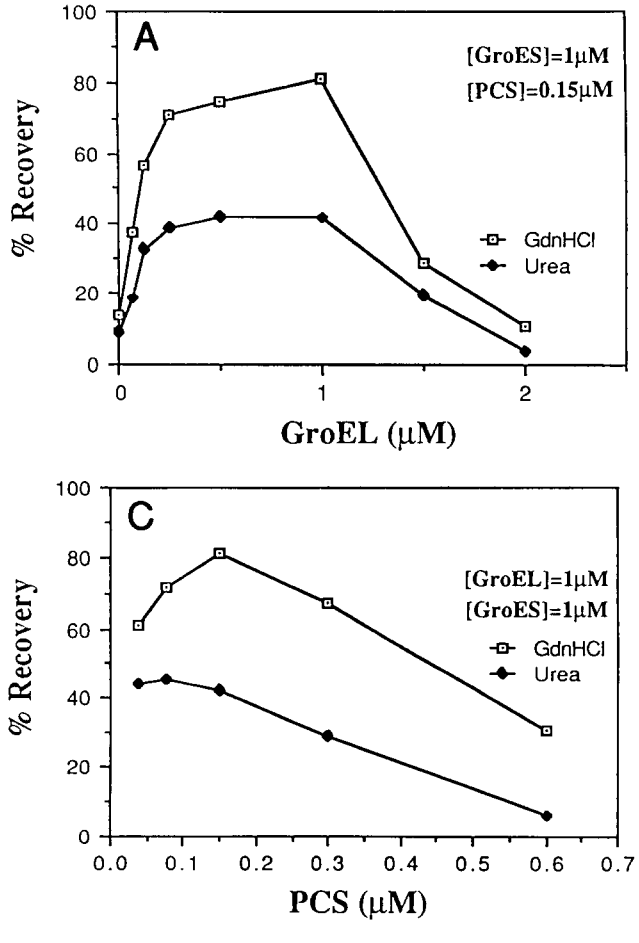


Fig. 4. Effect of CS monomer concentration on renaturation. Denaturation: The CS was denatured either by 6 M GdnHCl or by 8 M urea in 0.02 M $KHPO_4$ buffer, pH 7.5, containing 3 mM DTT and 2 mM EDTA. The incubation time was 30 min at room temperature. Renaturation: The denatured CS was diluted by 20-fold in 0.02 M $KHPO_4$ buffer, pH 7.5, containing 6 μM BSA and 0.5 mM OAA. Assay for recovery of the activity was made after the indicated incubation time at room temperature.

fect on protein renaturation (Tandon & Horowitz, 1987). However, in the present system lauryl maltoside had no effect on CS renaturation; nor did polyethylene glycol enhance renaturation.

Effect of the GroE proteins on CS refolding

If either urea- or GdnHCl-denatured CS is diluted into a mixture of GroEL, GroES, Mg-ATP, and K^+, then good recovery of activity is obtained. We find that recovery goes through an optimal GroEL concentration at constant GroES, and that there is an optimal denatured CS concentration at constant GroEL and GroES (Fig. 5A, C). On the other hand, the dependence on GroES at constant GroEL shows typical saturation curves (Fig. 5B). Without GroES, recovery is essentially the same as observed with no additions (<5%). Interestingly, recovery of activity with the GroE system is nearly twofold higher using GdnHCl-denatured CS than starting with urea-denatured CS. It is unlikely that covalent modification in the urea solution caused poor GroE-assisted refolding, as there was only a slight difference in BSA/glycerol-assisted refolding for the two denaturing conditions.



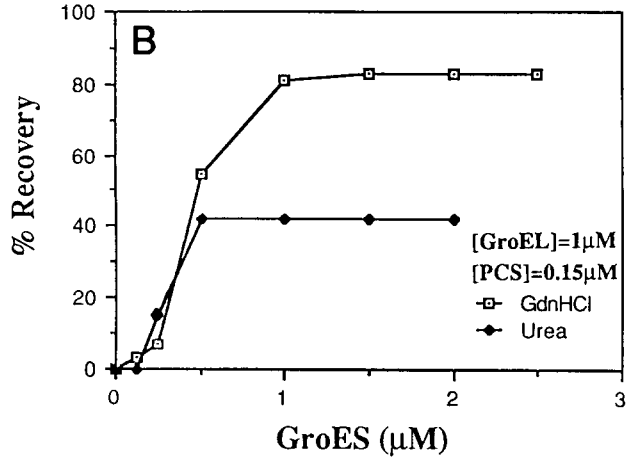


Fig. 5. Effect of concentration of GroEL (**A**), GroES (**B**), or CS (**C**) on recovery of CS activity after denaturation. Denaturation: The CS was denatured by 6 M GdnHCl in 0.1 M Tris/HCl buffer, pH 8.0, containing 20 mM DTT or in 8 M urea in the same buffer. The incubation time was 1 h at room temperature. Renaturation: The denatured CS was diluted by 100-fold in 0.1 M Tris/HCl buffer, pH 8.0, containing 10 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, and indicated concentrations of GroEL (14-mer), GroES (7-mer), or CS (monomer). Assay for recovery of the activity was made after 2 h incubation at room temperature.

The time-course for cpn-assisted recovery was not significantly different from that of recovery in non-GroE conditions. Inclusion of the non-GroE folding assistants did not improve the level of recovery obtained with the GroE system.

CS from *E. coli* was tested for cpn-assisted refolding as it is from the same organism as the cpns. *Escherichia coli* CS is a hexamer and is also denatured by GdnHCl and renatured in the presence of BSA, glycerol, or the complete GroE system.

We have also observed that addition of GroEL without GroES early in folding completely inhibits refolding of both GdnHCl- and urea-denatured CS in the presence of the non-GroE additives. Addition of GroEL after refolding has begun inhibits refolding to an extent that is time dependent. The time-course of loss of GroEL inhibition parallels the time-course of refolding. This result implies that GroEL acts on all incompletely folded CS molecules.

Discussion

The present study on the renaturation of CS has shown the following: (1) Refolding can be assisted by a number of separate mechanisms. The assistants to refolding include BSA, glycerol, OAA (a CS ligand), and the GroE system. (2) Chaperonin-assisted refolding is affected by the CS starting state, which differs following dilution from urea or from GdnHCl. We will now consider these points in detail.

Denaturation of CS

The denaturation of CS has been studied by several laboratories (Srere, 1966; Wu & Yang, 1970; Greenblatt & Sarkissian, 1972; West et al., 1990). West et al. (1990) showed that denaturation of CS in GdnHCl at concentrations less than 1.5 M, concentrations at which substantial activity is lost, followed by dilution, results in complete recovery of activity. The loss of activity in GdnHCl takes place at a much lower concentration (between 0 and 1 M) than changes in circular dichroism, fluorescence, and exposure of sulfhydryl groups, which all occur betweeen about 1.8 M and 3 M GdnHCl. Therefore, GdnHCl at low concentration causes a change in CS that is more subtle than global unfolding, which occurs at higher concentration.

We have shown earlier that four sulfhydryl groups are exposed when CS is treated with either GdnHCl or urea (Srere, 1966). A plot of SH exposure against denaturant concentration gave a slope of four for both GdnHCl and urea. Denaturation thus could be described as a one-step process in 6 M GdnHCl and 8 M urea. Furthermore, thermal denaturation of CS has been followed by change in circular dichroism, and the shape of this curve also corresponds to a one-step process (Zhi et al., 1991). These results indicate that the inactive species of CS generated by low GdnHCl concentration is not well populated in conditions that favor complete unfolding.

Monomers are the product of GdnHCl denaturation but not urea denaturation (Wu & Yang, 1970). Other workers also have noted differences between the final states of urea- and GdnHCl-denatured proteins (Pace et al., 1990). The interaction of the monomers in the CS dimer is a very strong one as judged from the X-ray crystallography of CS (Kinemage 1; Remington et al., 1982). The area involved in subunit interaction is large, and a large number of van der Waals contacts are apparent. In the absence of GdnHCl, CS does not easily dissociate into subunits. McEvily and Harrison (1986) have reported that CS can be dissociated into monomers if the CS concentration is low ($<10^{-7}$ M) and the pH is less than 7.0, and the extent of dissociation is sensitive to the nature and concentration of the buffer. In the urea denaturation conditions used here it is unlikely that dissociation into monomers occurred. Monomeric CS may be the species acted on most effectively by the cpn system, and this is reflected in the lower recovery of CS activity following denaturation in urea as compared to denaturation in GdnHCl.

Citrate synthase denaturation by heat or urea can be prevented by the addition of OAA (Srere, 1966). On the other hand, GdnHCl denaturation of CS is not affected by OAA. It is not likely that GdnHCl is a more effective denaturant as a result of its high ionic strength, because an increase in ionic strength decreases the effectiveness of (i.e., protects against) urea denaturation. It is apparent therefore that the mechanism of denaturation by GdnHCl differs from that by heat and urea, and heat and urea denaturation initiates at the active site of CS.

It has been shown spectroscopically that OAA causes a conformational change in CS (Srere, 1965), and Remington et al. (1982) have shown crystallographically that this change corresponds to the formation of a closed conformation from the open conformation by an 18° movement of the large domain toward the small domain in each monomer. This conformational change effectively shields the active site from the environment. A recent publication has shown that urea is an uncompetitive inhibitor for OAA in the CS reaction (Johnson & Srivastava, 1991). It is possible that a closed active site stabilizes the dimer against dissociation, but such a relationship would be subtle, since the active sites are distant from most of the CS dimer interface.

Renaturation of CS

Recovery of activity can be interpreted in at least two ways. First, one can assume that all enzyme activity is the result of fully active enzyme that has been restored identically to its original native structure. Second, it is possible that the activity observed is due to enzyme molecules that are only partially renatured with partial native en-

zyme activity. We assume that we are dealing with the first case based on the fact that low concentrations of GdnHCl, which do not produce changes in CS circular dichroism or fluorescence, cause marked loss of enzyme activity (West et al., 1990). Therefore, our recovery figures represent percent of original specific activity assuming the complete recovery of that number of enzyme molecules.

It should be noted that on dilution to the low protein concentrations used for renaturation, CS loses activity. The reason for this is not clear, and in the past we have used 1 mg/mL serum albumin as a protectant against dilution-inactivation of CS. Because other researchers have not commented on this phenomenon, it is difficult to know if the recoveries were based on controls in which CS activity is low as a result of this dilution-inactivation.

It was important to resolve the contradictory results in the literature on the renaturation of CS after GdnHCl denaturation. Greenblatt and Sarkissian (1972) reported that CS, which had been denatured in 6 M GdnHCl, could be 20% renatured by a 100-fold dilution of the denaturant. They reported that the presence of either 0.2 M KCl or 1 mM OAA increased the extent of renaturation to about 38%. They also reported that decreasing the CS concentration in the renaturation solution from 12.5 μg/mL to 3.1 μg/mL increased the rate and extent of renaturation to about 40%, even in the absence of KCl and OAA. These renaturation experiments were done with DTT present both during denaturation and renaturation. Nevertheless, we were unable to confirm these results under identical conditions, although a low level of recovery is seen.

West et al. (1990) also studied the GdnHCl denaturation–renaturation process of pig CS. If unfolding was performed in greater than 4 M GdnHCl, then no regain in activity was obtained following a 60-fold dilution. The principal difference between this experiment and that of Greenblatt and Sarkissian is that West et al. (1990) denatured CS in the absence of DTT, whereas Greenblatt and Sarkissian (1972) denatured CS in the presence of DTT.

What are the mechanisms of BSA-, OAA-, glycerol- and GroE-assisted refolding? It is generally believed that the concentration of denatured protein in renaturing conditions must be sufficiently low to reduce nonspecific aggregation, as this process is highly concentration dependent. Buchner et al. (1991) studied the aggregation of denatured CS by light scattering techniques and saw little evidence for aggregation at CS monomer concentrations less than 0.1 μM. We ran a number of experiments below this concentration and still obtained little recovery unless BSA, OAA, or glycerol was present. The data indicate that an irreversible process occurs upon dilution. That process could be aggregation (to a particle size that is below detection with the method of Buchner et al.) or formation of an incorrectly folded structure. Each of the three non-GroE folding assistants probably acts by a different mechanism. OAA may bind to a partially formed active site, which then acts as a folding nucleation point (see Kinemage 2), whereas BSA may stabilize structures that approach the native conformation. The action of glycerol may be similar to that of BSA, as substances like polyethylene glycol, which mimics the water exclusion and viscosity effects of glycerol, do not enhance renaturation.

The SH requirement for recovery following denaturation in GdnHCl probably results from a strong tendency to form intrachain disulfide bonds that interfere with correct folding. In urea, because the denatured protein is still dimeric, such interactions may be more difficult to achieve, thus explaining the reduced effect of DTT in urea.

Refolding denatured CS by the GroE system

Our results demonstrating folding assistance by the GroE system are consistent with the report of Buchner et al. (1991), except that we observed almost no recovery without GroES. The pronounced difference in the optimal recovery (28% for Buchner et al. and 82% reported here) strongly emphasizes the effect of GroES in our experiments. We conclude that GroES *is* essential for GroE-assisted refolding of CS, in contrast to the suggestion by Buchner et al. (1991) that it is not. In addition, we show that the dependence on GroEL and GroES concentrations is similar to that reported by Goloubinoff et al. (1989) for Rubisco in that optimal reconstitution is seen at about equimolar GroEL and GroES, and reconstitution is inhibited when the GroEL concentration exceeds that of GroES. Recently, Mendoza et al. (1991a) have shown that the GroE system will facilitate the refolding of rhodanese, a monomeric mitochondrial protein. They also show that the spontaneous refolding of rhodanese is inhibited by GroEL. For CS, we see that refolding assisted by BSA is inhibited by GroEL. Martin et al. (1991) also reported that GroEL alone inhibited the spontaneous refolding of rhodanese as well as dihydrofolate reductase.

The model described by Martin et al. (1991) proposes a "molten globule"-like intermediate at the GroEL surface, which proceeds to native protein by the action of GroEL with GroES and ATP. Mendoza et al. (1991a) propose binding of hydrophobic regions of denatured proteins to GroEL where renaturation will occur. Our present data cannot extend this hypothesis, but in the case of urea-denatured CS where a dimer exists, the GroE system is not as efficient as it is with GdnHCl-denatured CS monomers.

Implications

Our results suggest that there are many factors that can influence the adoption of native structure from denatured states. This observation accounts for the diversity of ap-

parent mechanisms by which assisted renaturation occurs. These in vitro findings foreshadow a multitude of mechanisms for chaperone-facilitated renaturation in vivo. Furthermore, many species such as cofactors, substrates, and other (non-chaperone) proteins will undoubtedly modulate folding in the cellular context. The observation that assistants do not enhance cpn-facilitated refolding suggests alternate paths to a native protein in vivo.

Materials and methods

Materials

Pig heart CS (EC 4.1.3.7) was obtained from Boehringer Mannheim Corp. (Indianapolis, Indiana). Fumarase (EC 4.2.1.2), lysozyme (EC 3.2.1.17), malate dehydrogenase (mitochondrial) (EC 1.1.1.37), urea, GdnHCl, OAA, 5,5′ dithiobis-(2-nitrobenzoic acid) (DTNB), DTT, and BSA were from Sigma Chemical Co. (St. Louis, Missouri). Acetyl coenzyme A was prepared by the method of Simon and Shemin (1953). All other chemicals used were of the highest purity available. GroEL and GroES were isolated from *E. coli* as previously described (Landry & Gierasch, 1991).

Denaturation

Pig heart CS (M_r = 100,000) was denatured either by incubating the enzyme in buffered (exact conditions described in Table 1 and in the figure legends) 6 M GdnHCl or 8 M urea at room temperature for 30–60 min. Urea solutions were always prepared on the same day as their use.

Renaturation

Renaturation was initiated by diluting the denatured enzyme solution 20–100-fold (depending on the experiment) into buffer with various additions. During the course of these studies, we have used either 0.02 M $KHPO_4$ (pH 7.5) or 0.1 M Tris/HCl buffer (pH 8.1) as buffers for denaturing and refolding steps. Only small differences in recovery were observed when the same experiments were run in the two different buffers or when 1:20 dilutions were compared to 1:100 dilutions. The exact conditions for denaturing and refolding are given in Table 1 or in the figure legends. Because there is some variability in recoveries from day to day, we investigated the result of varying the dilution conditions. Usually we added 1 μL of denatured CS to 100 μL of renaturing solution in a 1.5-mL Eppendorf tube that was being vortexed. We have tried dilutions of 5 μL to 500 μL and dilutions in which the renaturing solution was added to the denatured CS. No differences were observed as long as instantaneous mixing occurs, but some care must be taken at this step. The concentrations of urea or GdnHCl (~0.5–1 mM) that are present in the final assay cuvette have no effect on enzyme activity. It has also been demonstrated that the concentrations of urea or GdnHCl that are in the refolding solution (0.04 and 0.03 M) do not cause denaturation of CS.

Enzyme assay

The enzyme activity was assayed by measuring the decrease in absorption at 233 nm due to the cleavage of acetyl CoA and the utilization of OAA (Srere & Kosicki, 1961). This assay gives identical results with the DTNB method (Srere et al., 1963) in calculating the specific activity of the enzyme. The DTNB method cannot be used if there is a high concentration of DTT in the experiment.

Protein determination

The M_r's used are: CS (a dimer) 10^5; BSA 6.9×10^4; GroEL (a dimer of heptamers) 8×10^5; GroES (a heptamer) 7×10^4. Protein was determined using the method of Bradford (1976). For BSA this was standardized gravimetrically; for CS this was standardized using A_{280} (Singh et al., 1970); and for GroEL and GroES this was standardized by amino acid analysis.

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Figure added in proof (for interactive color version, see file \KINEMAGE\Zhi.kin on the April 1992 *Protein Science* Diskette Appendix). Alpha-carbon trace of core domains in a single subunit of citrate synthase (Brookhaven file 4CTS; Remington et al., 1982). The amino-terminal 64 residues and carboxy-terminal 21 residues contact mainly the other subunit and are not shown. OAA (stippled surface) at the active site is near the center of the compact core domain 2 (thin line, residues 222–416). Core domain 1 (thick line, residues 65–222) wraps around the outside. This view makes it plausible that OAA could assist folding by helping organize a folding nucleus around the active site.