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Control of aggregation in protein refolding: A variety of surfactants promote renaturation of carbonic anhydrase II

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Abstract

The denaturation and renaturation of carbonic anhydrase II (CAII) has been studied in several laboratories. Both thermodynamic and kinetic evidence support the existence of at least two intermediates between denatured and native protein. Previous studies have shown that on rapid dilution of a CAII solution from 5 M to 1 M guanidinium chloride, aggregation strongly competes with renaturation at higher protein concentrations, suggesting an upper limit for [CAII] of ~0.1%. Our experiments show 60% renaturation at 0.4% [CAII] and that aggregate formation is partially reversible. This yield can be substantially increased by several surfactant additives, including simple alkanols as well as micelle-forming surfactants. Effective surfactants (promoters) act by suppressing initial aggregate formation, not by dissolving aggregates. Promoters act on either the first folding intermediate (I_1) or oligomers thereof. Eight of the 18 surfactants examined showed promoter activity, and no correlation was evident between promoter activity and chemical structure or surface tension lowering. These results indicate discrimination (molecular recognition) by I_1 and/or its oligomers.

Keywords: protein folding; surfactants; suppression of protein aggregation

Protein refolding, as in the renaturation of solubilized inclusion bodies, is often frustrated by competing reactions leading to aggregation and low recovery of soluble native protein (Saxena & Wetlaufer 1970; Zettlmeissl et al., 1979; Brems, 1988; and numerous papers in the recent volumes edited by Georgiou & De Bernardez-Clark, 1991; and by Cleland, 1993). Refolding is usually carried out by manipulating the concentration of denaturant in the refolding solvent and/or the kinetics of denaturant removal. This process may be modeled on the assumption that a species intermediate between denatured and native protein, sometimes called a molten globule (Ptitsyn & Uversky, 1994), exposes a greater-than-native fraction of nonpolar residues to solvent, leading to increased intermolecular association. Variations on this model have been suggested (Rudolph & Buchner, 1991; De Young et al., 1993). One obvious practical approach to this problem is high dilution of the protein. Although this can substantially improve yields, the resulting large volumes complicate subsequent recovery of native protein. We have here explored several parameters that affect refolding of a model protein, including increasing the solubility of folding intermediates by surfactants that do not denature the native protein. Obvious surfactant candidates were drawn from the membrane biochemists' armamentarium; less obvious candidates from more fundamental considerations (Tang & Deming, 1983).

In this study we employ the extensively studied refolding of carbonic anhydrase (CAII) in dilute guanidinium chloride (GdmCl) solutions (Yazgan & Henkens, 1972; Wong & Tanford, 1973; Ikai et al., 1978; Cleland & Wang 1990a, 1990b; Semisotnov et al., 1990; Cleland et al., 1992).[1] These studies have shown the existence of two intermediates between denatured and native protein. The earlier of these is formed very rapidly and shows evidence of compactness and secondary structure but lacks some of the specific three-dimensional structure of the native molecule. This early intermediate also binds hydrophobic fluorescent probes, thereby demonstrating nonpolar surfaces not present in the native protein. The first intermediate can isomerize to a second intermediate and then to native protein but alternatively is capable of forming micron-size aggregates via oligomer formation (Cleland & Wang, 1990a). This early folding intermediate has been shown to bind polyethylene glycol reversibly and thereby inhibit aggregate formation (Cleland & Wang, 1990b). Because polyethylene glycol has a peculiar structure for a sur-

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[1] Carbonic anhydrase II, as provided by Sigma Chemical Co., was formerly labeled carbonic anhydrase B. Therefore, this study and those referenced here are assumed to have studied the same enzyme.

factant, it was of interest to explore a variety of other surface-active materials. With the objective of refolding at relatively high protein concentrations, we carried out our survey to satisfy that condition. In addition to measuring the kinetics, we have monitored the turbidity of the refolding solutions. Our survey shows that several surfactants, including polymeric, micelle-forming, and nonmicelle-forming, acted as renaturation promoters.

Our results are largely consistent with the previously proposed folding scheme for folding plus off-pathway aggregation (Cleland & Wang, 1990a). However, we find that aggregate formation is partly reversible over the time course of typical refolding experiments. Examining the mechanism of surfactant promoters, we find that they act on soluble folding intermediates and not by dissolution of aggregates. We also show that, even in the absence of surfactants, refolding can occur at much higher protein concentrations than would be predicted by earlier work.

Results

When a 5 M GdmCl solution of CAII is rapidly diluted with buffer to 1 M or lower, rapid recovery of enzymic activity follows (Fig. 1A). It is evident that higher plateau yields are obtained with higher GdmCl concentrations in the folding solvent. The kinetics are, on close examination, biphasic, and the data are insufficient to determine how much the initial rates depend on GdmCl concentration. These results are qualitatively similar to those previously reported for CAII refolding at similar [GdmCl] and [CAII] (Cleland & Wang, 1990b). However, that work did not extend observations beyond 30 min and therefore in some cases did not show the biphasic character. The effect of increasing protein concentration in decreasing both the rate and recovery is shown in Figure 1B. The lowest concentration of CAII refolds with a progress curve that fits first-order kinetics (plot not shown), whereas the two higher concentrations do not. Again, as in Figure 1A, the biphasic nature of the refolding at higher concentrations is seen. We do not find any previous studies indicating that substantial yields of renatured protein are obtainable at such high (4.0 mg/mL) CAII concentrations.

It was evident by visual observation that large aggregates form at protein concentrations >1 mg/mL (see lowest curve in Fig. 1B). We considered it useful to make quantitative measurements of the apparent absorbance in a nonabsorbing spectral region and to plot these on the same time scale as that of the previous figure. The results (Fig. 2A) show clearly that, within the dead time of mixing, the maximum apparent turbidity is reached, and that this decays monotonically over the time course of the experiment. This result is somewhat surprising in light of light-scattering results (Cleland & Wang, 1990a), which show large particles increasing with time—albeit at much lower protein and denaturant concentrations. Putting aside for the moment the results with *n*-hexanol additive, we see ~30% decrease (initial to final observation time) in the turbidity at 4 mg/mL CAII, and about the same percentage decrease for the 2-mg/mL solution. An obvious although not unique interpretation of the decline in turbidity is that aggregate formation is partly reversible.

The necessity of Zn^{2+} for rapid refolding of CAII was demonstrated by Yazgan and Henkens (1972). In some of the refolding studies of Wang and Cleland (1990a, 1990b), millimolar EDTA was apparently present in the refolding solvent. Our control experiments (not detailed here) with millimolar EDTA in the refolding solvent showed no regeneration of enzymic activity; presumably Zn^{2+} is preferentially associated with EDTA.

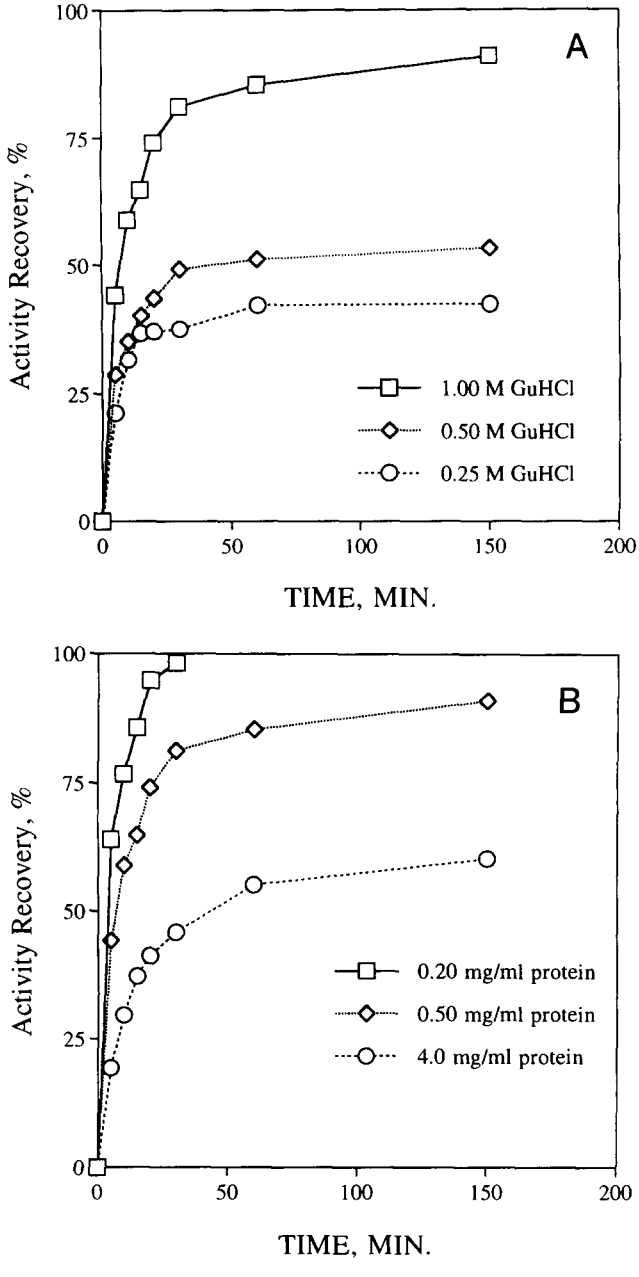

Fig. 1. A: Refolding of CAII with different concentrations of GdmCl. □, 1.00 M; ◇, 0.50 M; ○, 0.25 M. Refolding solvents also contained 50 mM Tris-sulfate, pH 7.5. CAII concentration was 0.50 mg/mL. **B:** Refolding of CAII with different concentrations of CAII. □, 0.20 mg/mL; ◇, 0.50 mg/mL; ○, 4.0 mg/mL. Refolding solvent was 1.00 M GdmCl, containing 50 mM Tris sulfate, pH 7.5.

While considering the suppression of refolding by EDTA, we carried out a comparison of turbidity with and without 4 mM EDTA in the 1 M GdmCl refolding solvent. The results of one such pairwise comparison are shown in Figure 2B. These show that turbidity gradually increases in the presence of 4 mM EDTA, contrasting with the gradual decrease found in the absence of EDTA. Replicate experiments showed the same qualitative dif-

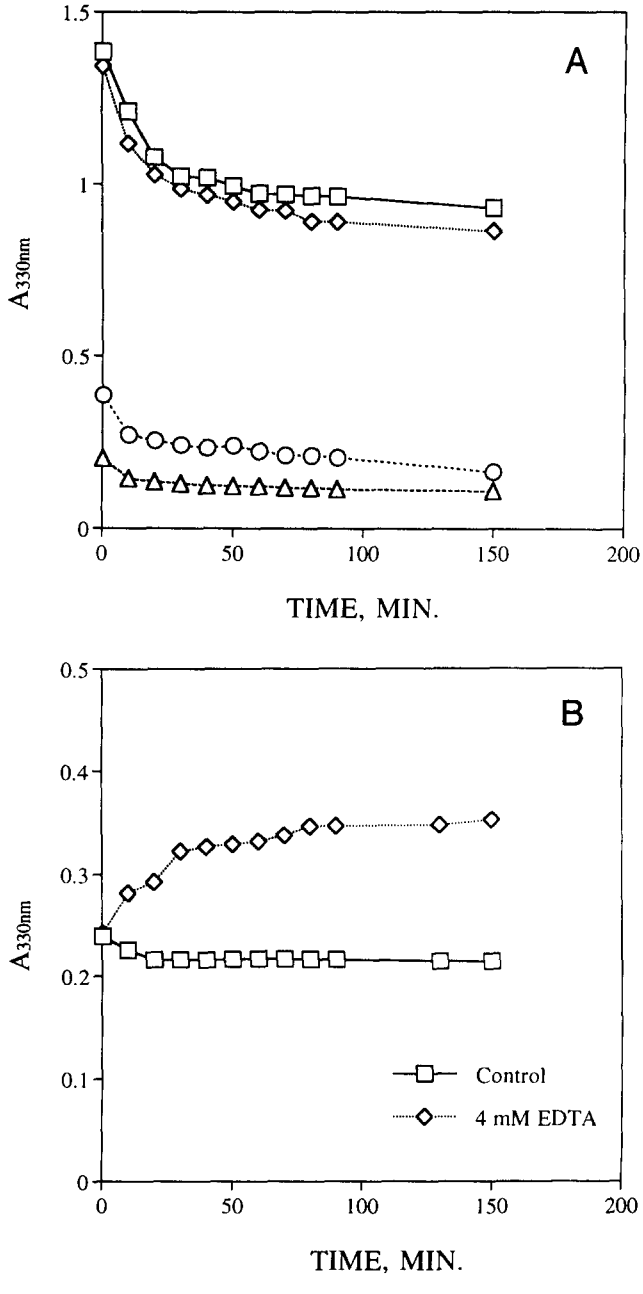

Fig. 2. A: Turbidity development during CAII refolding in 1.00 M GdmCl. Upper curves: □, 4.0 mg CAII/mL; ◇, 4.0 mg CAII/mL with 0.01% *n*-hexanol. Lower curves: ○, 2.0 mg CAII/mL; △, 2.0 mg CAII/mL with 0.01% *n*-hexanol. Refolding solvent contained 50 mM Tris sulfate, pH 7.5. **B:** Turbidity development during CAII refolding, ◇, in the presence of 4 mM EDTA; □, in the absence of EDTA. CAII concentration, 2.0 mg/mL. Refolding solvent was 1.00 M GdmCl containing 50 mM Tris sulfate, pH 7.5.

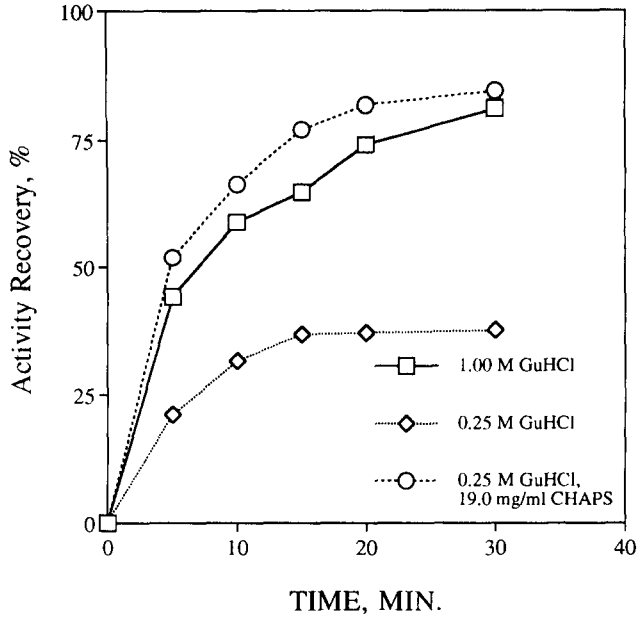

Fig. 3. Enhancement of CAII refolding by CHAPS, ○, in the presence of 31 mM CHAPS; ◇, in the absence of CHAPS. CAII concentration, 0.50 mg/mL. GdmCl concentration, 0.25 M. Refolding solvent contained 50 mM Tris sulfate, pH 7.5. Refolding of the same concentration of CAII in 1.00 M GdmCl, □, is shown for comparison.

ference, although the turbidity values were quantitatively different, presumably because manual mixing is not highly reproducible.

In an exploratory experiment with low concentrations of both CAII and GdmCl in the refolding solvent, the surfactant CHAPS is clearly seen (Fig. 3) to increase the rate and the yield of renatured enzyme. As shown in this figure, the activity yield at 30 min with CHAPS was 81%, compared with 37% for a regeneration identical but lacking CHAPS. The concentration of CHAPS employed in this experiment, at 31 mM, is substantially greater than the reported critical micelle concentration (CMC) values in water (Hjelmeland et al., 1983; Stark et al., 1984) and the value we obtained in 1 M GdmCl (see Materials and methods).

These results with CHAPS were strongly encouraging: a twofold increase in yield in a reasonable time. However, they were carried out at the relatively low protein concentration of 0.50 mg/mL; a practical solubilizing additive should be effective at much higher protein concentrations. Therefore an eightfold higher CAII concentration was employed in an examination of refolding in the presence of several alkanols. Figure 4 shows the activity recovery after 150 min refolding of CAII at 4.0 mg/mL, with several alkanols as additives. The complete timecourse of the refolding in the absence of additive is the lowest curve in Figure 1B. Within experimental error, the C_3 and C_4 alcohols showed no effect on recovery, neither inhibiting nor enhancing. The next three higher alcohols show substantial enhancement of recovery over a wide range of concentration. *n*-Pentanol and *n*-hexanol show a similar pattern, the latter enhancing recovery by 25% above the control with no additive. Both pentanol and hexanol show saturation behavior, with [*n*-hexanol] = 10^{-5} M and [*n*-pentanol] = 2×10^{-4} M at half-saturation. The single point at the highest *n*-pentanol concentration may be spurious; it approaches the solubility limit of the alcohol. Similar refolding experiments were carried out with *n*-octanol as additive; activity recovery was 60% ± 3% (no different than control) over concentrations ranging from millimolar to micromolar.

Another group of putative solubilizing additives was examined, which included amphiphilic polymers and micelle-forming surfactants. The results are seen in Figure 5, which shows at least

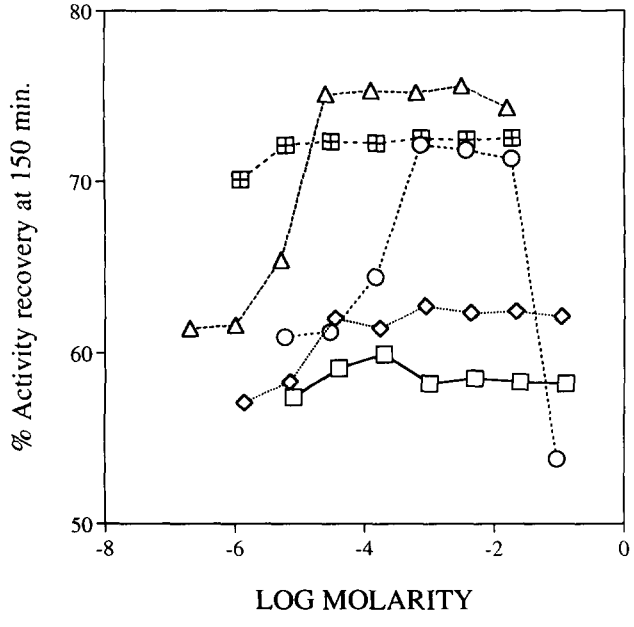

Fig. 4. Recovery of CAII activity after 150 min refolding, with various surfactant additives in the refolding solvent. □, *n*-propanol; ◇, *n*-butanol; ○, *n*-pentanol; △, *n*-hexanol; ⊞, cyclohexanol. CAII concentration, 4.0 mg/mL. GdmCl concentration, 1.00 M. Refolding solvent also contained 50 mM Tris sulfate, pH 7.5. Each point on the graph represents duplicate determinations of activity. Control recovery (in the absence of any surfactant additive), 60 ± 3%.

a slight enhancement in renaturation at some concentration by all these additives. Triton X-100 and CHAPS show strongly concentration-dependent enhancement/inhibition. $C_{12}E_8$ shows the greatest enhancement of all these additives (essentially the same as *n*-hexanol) over a 1,000-fold concentration range. As CHAPS concentration is increased well above its CMC, its effect becomes inhibitory; in contrast, $C_{12}E_8$ shows no discontinuity across its CMC ($\sim 10^{-4}$ M). Triton X-100 is inhibitory at its highest concentration (~0.1 M), but this is $\sim 10^3 \times$ higher than its CMC (Brito & Vaz, 1986). Polyvinylpyrrolidone increases the yield only marginally above that of the control, whereas polyethylene glycol substantially increases the yield over a broad concentration range. It should be noted that Cleland and Wang (1990b) have shown that polyethylene glycol promotes CAII refolding at much lower protein concentrations.

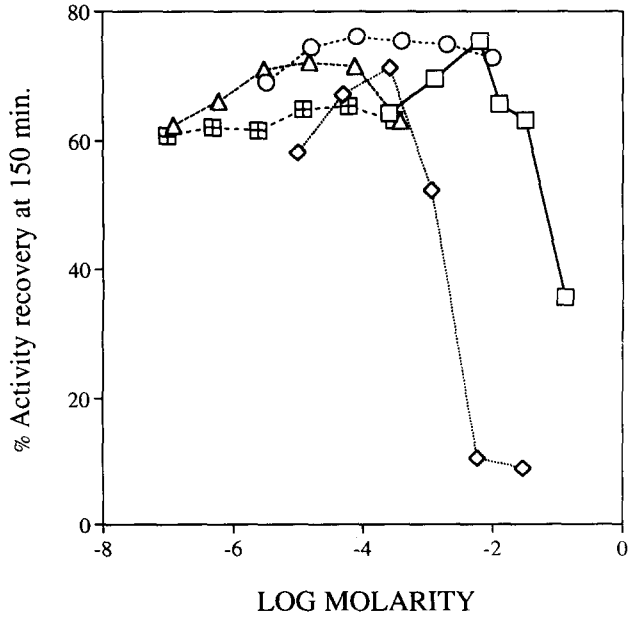

Fig. 5. Recovery of CAII activity after 150 min refolding, with various polymeric or micelle-forming surfactants. Surfactants added: □, Triton X-100; ◇, CHAPS; ○, $C_{12}E_8$; △, PEG-8000; ⊞, polyvinyl pyrrolidone. Remaining details are the same as for Figure 4.

Seven additional surfactants were tested with the same system that provided the data for Figures 4 and 5. Of these, only cycloheptanone showed enhanced yield, with a yield versus concentration pattern similar to that of cyclohexanol (Fig. 4). Cyclohexyl ethanol, benzyl alcohol, *N*-octyl pyrrolidone, *n*-octyl β-D-glucopyranoside, nonanoyl-*N*-methylglucamide, and Zonyl FS-300 all produced control yields of enzyme activity at 150 min over a substantial concentration range.

Having shown evidence of enhancement of native protein formation by these simple alcohols, we turned to examine the effects on the inactive fraction, as expressed in the turbidity. One can see that turbidity declines monotonically from an early maximum in the presence or absence of hexanol, the most effective surfactant in the group shown in Figure 4. All four curves in Figure 2A show a substantial decrease in turbidity with time. Hexanol decreases the observed turbidity over the whole time course, but the percent decrease is clearly greater at the lower protein concentration (Fig. 2A, lower pair of curves).

With which intermediate species do the additives interact to enhance the final yield? Refolding experiments were carried out in which three progress curves for CAII renaturation at moderate protein concentration and low GdmCl concentration are compared (Fig. 6). In the first of these three, $C_{12}E_8$ was present from zero time in the refolding solvent; in the second, $C_{12}E_8$ was added 10 s after zero time; and in the third, no additive was present (control). As Figure 6 shows, when $C_{12}E_8$ is present at the start of the refolding, both the initial rate and the yield of native protein are substantially greater than in the control. When the addition of $C_{12}E_8$ is delayed to 10 s after the start of refolding, the progress curve is superimposed on that of the control. In logically similar experiments, effective surfactants were added to control renaturations 150 min after initiation of refolding. Neither *n*-hexanol (10^{-3} M) nor $C_{12}E_8$ (10^{-4} M) added at 150 min led to an increase above the 60% control yield from 150 to 250 min. When the same concentrations of the respective surfactants were present from the start of refolding, both led to yields of 75–77%. Both of these "delayed surfactant addition" experiments fit the idea that the surfactants act only on very early intermediates and do not facilitate dissolution of aggregates.

To examine the question further, "With what intermediates do the effective surfactants interact?," refolding experiments were carried out at low protein concentrations, which should favor monomeric over oligomeric species. In Figure 7A we see that, with no additives, refolding at 0.20 mg CAII/mL is slower than at 0.07 mg/mL. Oligomerization of an intermediate at the higher concentration probably accounts for this slowing. Surprisingly, both $C_{12}E_8$ and *n*-hexanol reduce the early rate of refolding. Because this is in contrast with the increase in 150-min yield seen (Figs. 4, 5) with these surfactants at 4.0 mg CAII/mL, it was desirable to confirm the 4-mg/mL finding. Figure 7B

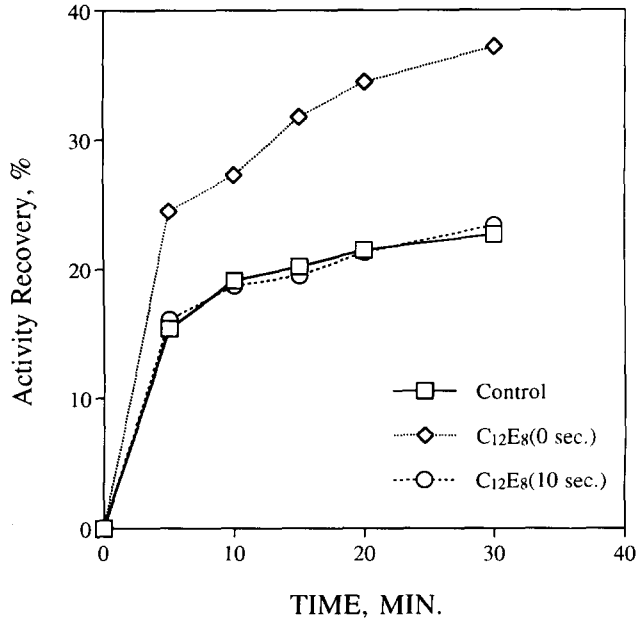

Fig. 6. CAII refolding with, ◇, $C_{12}E_8$ added at zero time compared with, ○, $C_{12}E_8$ added 10 s after dilution of protein to 0.25 M GdmCl; □, folding with no surfactant. CAII concentration, 0.50 mg/mL; GdmCl, 0.25 M; $C_{12}E_8$, 0.40 mM. Refolding solvents also contained 50 mM Tris sulfate, pH 7.5.

shows progress curves at [CAII] = 4 mg/mL with and without $C_{12}E_8$. The results show that the presence of $C_{12}E_8$ has no effect on the early rate and confirm that this surfactant increases the longer time yield.

Discussion

Our results can usefully be discussed in terms of the reaction scheme shown in Figure 8, based on the work of Cleland et al. (1992) and several other laboratories (Wong & Tanford, 1973; Ikai et al., 1978; Stein & Henkens, 1978; Dolgikh et al., 1984; Cleland & Wang, 1990a; Semisotnov et al., 1990). Although some of our results are not fully consistent with this scheme, it provides a very useful reference framework. Figure 1A shows that the practical recovery of CAII activity at 150 min decreases with decreasing [GdmCl]. Qualitatively this result is in agreement with that of Cleland and Wang (1990b). The increasing yield with increasing [GdmCl] is consistent with the ability of this denaturant to solubilize molecules with nonpolar surfaces (Wetlaufer et al., 1964). A further increase in [GdmCl] above 1.00 M would be counterproductive, because the equilibrium denaturation of CAII increases rapidly with increasing [GdmCl] above 1.00 M (Yazgan & Henkens, 1972; Wong & Tanford, 1973; Ikai et al., 1978). Figure 1B shows that increasing [CAII] both slows the rate of native enzyme formation and decreases the yield. Refolding yields of native protein are commonly found to decrease with increasing protein concentration. This is reflected mechanistically in Figure 8, where increasing $[I_1]$, the obligatory first intermediate, favors off-pathway oligomer formation, leading to aggregation.

However, Figure 1B also shows the surprising result that at the highest [CAII], 4 mg/mL, 60% refolding occurs in 150 min.

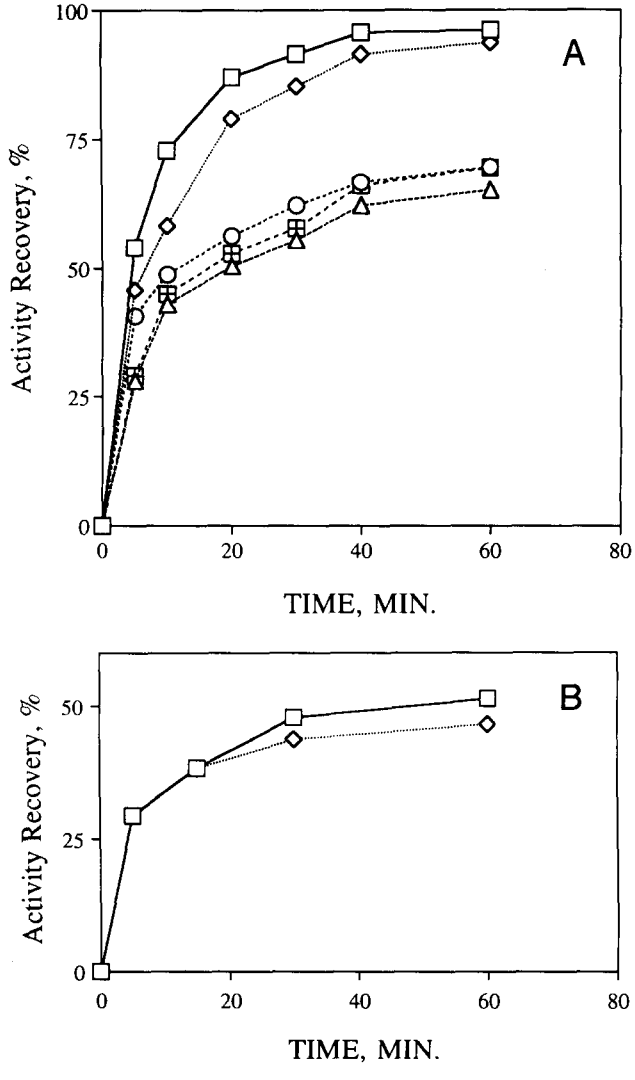

Fig. 7. A: Refolding of CAII at low protein concentrations. The top two curves were carried out at 0.07 mg/mL CAII in 1.00 M GdmCl: □, control containing no surfactant; ◇, containing 0.40 mM $C_{12}E_8$. The next three curves from the top were carried out in the same solvent, with CAII concentration, 0.20 mg/mL; ○, control with no surfactant; ⊞, 0.40 mM *n*-hexanol; △, 0.40 mM, $C_{12}E_8$. Duplicate refolding experiments in these three solvents produced the same order of reaction velocities: ○ > ⊞ > △. **B:** These two curves, at higher protein concentration than in A, are shown for comparison to the low-concentration groups. CAII concentration was 4.0 mg/mL; GdmCl, 1.00 M; □, 0.4 mM $C_{12}E_8$; ◇, no surfactant. Results for the 5-min and 15-min time points are superimposed for the two regenerations. Refolding solvents in both A and B contained 50 mM Tris sulfate, pH 7.5.

This is surprising in light of Cleland and Wang's (1990a) description of a "refolding regime," which has an upper limit of ~1.2 mg/mL for CAII. It appears that these workers did not actually test CAII concentrations >1.0 mg/mL nor refolding times longer than 30 min. It should be noted that Cleland and Wang defined the "refolding regime" as a two-dimensional surface defined by GdmCl and CAII concentrations such that no large (micron-sized) aggregates form during the course of the reaction. Of course, with 4 mg/mL CAII, we have abundant aggregate formed—both by visual inspection and by simple turbidity

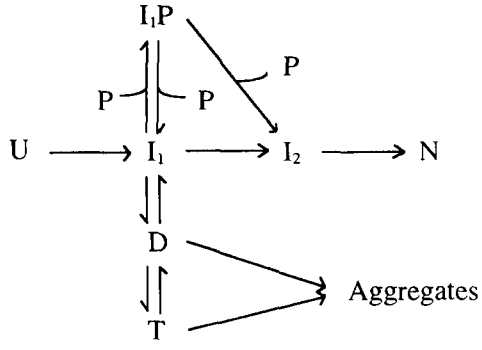

Fig. 8. Model for refolding of CAII in GdmCl, after Cleland et al. (1992). When the unfolded protein, U in 5 M GdmCl, is rapidly diluted to GdmCl ≤ 1 M, the first intermediate, I_1, is formed rapidly ($t_{1/2} <$ 1 s). At high dilution of CAII, I_1 isomerizes to I_2 ($t_{1/2} = 120$ s), which subsequently rearranges ($t_{1/2} = 550$ s) to form N, the native protein. At higher protein concentrations, self-association of I_1 yields dimer (D) and trimer (T), which in turn can irreversibly form micron-size aggregates. When a surfactant promoter P (polyethylene glycol was used by Cleland et al., 1992) is added to the system, it can reduce aggregate formation by reversibly binding I_1, competing with dimerization. The kinetic parameters cited were obtained for refolding in 0.60 M GdmCl at 23 °C (Semisotnov et al., 1990) and are noted only to provide order-of-magnitude estimates for the range of conditions encountered in the present work.

measurements (Fig. 2A). However, if one is willing to separate the aggregated protein, by filtration or centrifugation, a 60% yield is substantial. Further, we suppose that the aggregated material might be collected, redissolved at high [GdmCl], and put through another refolding cycle. Because of the expense of CAII, we did not attempt to refold CAII at even higher concentrations, but there is no reason to believe that this could not be done with substantial recovery of native enzyme.

Because the analytical methods used for Figures 1 and 3 did not stop refolding during assay (see *Methods*), quantitative analysis of the kinetics is inappropriate.

Refolding of more concentrated CAII solutions shows (visual inspection) the formation of strong turbidity immediately following dilution (5 M GdmCl → 1.00 M). When turbidity is monitored by apparent absorbance, the signal decays monotonically. Figure 2A suggests that turbidity has a protein concentration dependence to ~1.8 power. Because the manual mixing of small volumes is not highly reproducible, and because Beer's Law does not hold for turbidity, this can only be a very crude guide, but it does appear to be in rough agreement with the idea that the initial turbidity depends on a reaction that has the same concentration dependence as dimerization.

More important is the observation that, from an early maximum, the turbidity decreases with time. This is in contrast with the findings of Cleland and Wang (1990a), who showed by Quasi-Elastic Light Scattering (QLS) that the population of micron-size particles increased with time. Although their observations were made at lower concentrations of CAII (0.50 mg/mL) and GdmCl(0.70 M) than shown in Figure 2A, it is surprising that the trend should be so different. This unexpected finding prompted us to carry out comparative measurements of turbidity with and without EDTA in the refolding solvent. The results (Fig. 2B) show that the presence of EDTA gives rise to a slow monotonic increase in turbidity. We cannot draw a firm conclusion on this issue, but it appears possible that at least some of Cleland and Wang's QLS refolding experiments were carried out in the presence of EDTA. If this were so, the whole folding reaction course is very different, as shown by Yazgan and Henkens (1972), comparing the refolding rates of zinc-free enzyme and holoenzyme.

Surfactant additives

General experience in membrane biochemistry shows that surfactants bearing net charge bind strongly to proteins and tend to denature them. Surfactants of zero net charge are preferred for solubilization without denaturation. With this in mind, we chose CHAPS, a dipolar ion derivative of cholic acid, as an additive in our initial experiments. Control activity assays with native CAII showed no interference by CHAPS. Based on the report of Tandon and Horowitz (1987) that refolding of rhodanese is assisted by micelles of dodecyl maltoside, but not by monomers, we chose a concentration substantially higher (31 mM) than the reported CMC of CHAPS (4–8 mM, Hjelmeland et al., 1983; Stark et al., 1984). Under low-yield refolding conditions, the addition of CHAPS doubled the 30-min yield of active enzyme, which appears to have reached a plateau in the absence of CHAPS (Fig. 3). This was an encouraging result but also a time to reassess our objectives.

We might have chosen to use the control conditions of Figure 3 as reference for comparing other surfactant additives. Instead, we chose to employ much higher protein concentrations (4.0 mg/mL) and higher GdmCl (1.00 M) concentrations. Increasing both these concentrations is consistent with a general objective for a preparative process: to carry out high-yield refolding without excessive protein dilution (=volume increase). Here the advantage of approaching practical process conditions outweighed the appeal of economy resulting from working with dilute solutions of a moderately expensive enzyme. However, the issue of economy of both time and materials was reasserted in choosing to survey a large number of surfactant additives by only measuring recoveries at 150 min, instead of measuring complete progress curves.

What Figures 4 and 5 display, then, are the end-point yields of enzyme activity: the limiting percentage of refolded protein formed, as affected by various surfactant candidates to be promoters. First of all, the results of this survey show that roughly half of the 18 surfactants added show some enhancement of yield. Although the interactions of the surfactants are almost certainly with one or more partly organized folding intermediates (see below), the interacting protein clearly can discriminate at the molecular or oligomer level, even between similar surfactants. Considering the possibility that the effects of surfactants in 1.00 M GdmCl might be very different from those in water, we compared the surface tensions of several alcohols in the two solvents. These alcohols (see *Methods*) showed virtually the same surface tension lowerings in the two solvents.[2]

It is remarkable that several promoters are effective at very low concentrations. For example, *n*-hexanol is still maximally

[2] Although, strictly speaking, we should attempt to correlate interfacial tensions with folding enhancement, such measurements would require a suitable model for the surface of the I_1 intermediate. Lacking such a model, we rely on air/liquid surface tensions to provide at least a qualitative guide.

effective at 5×10^{-5} M, whereas [CAII] = 1.3×10^{-4} M. This indicates a stoichiometry of 1 hexanol to 2 or 3 CAII monomers, and the concentration dependence of enhancement looks to be saturable—note the plateau from 5×10^{-5} to 5×10^{-3} M *n*-hexanol.

This stoichiometry is inconsistent with the mechanism shown in Figure 8, which has promoters interacting with monomeric I_1. However, if the promoter were interacting mostly with dimers and trimers, the stoichiometry could be satisfied. The experiments of Figure 7 were carried out to address this question. Refolding at low [CAII] should increase the percent monomer (I_1) in the pathway. Our expectation was that, if promoters interact with monomer and not with dimer and trimers, a promoter-altered refolding would still be seen at very high dilution. What Figure 7A shows is that the promoters affect the refolding to about the same extent at [CAII] = 0.20 mg/mL and at 0.07 mg/mL. Our attempts to make the comparison at still lower [CAII] were inconclusive due to high signal/noise in the assays.

The results in Figure 7A are consistent with, but not decisive evidence for promoter interaction with dimers and trimers.

Many of the promoters do not form micelles, and the most effective micelle-forming promoter, $C_{12}E_8$, is equally effective above and below its CMC (1.7×10^{-4} M in 1.00 M GdmCl). Therefore, under the conditions of Figures 4 and 5, micelle formation is not a requirement and, in particular cases (CHAPS and Triton X-100), may inhibit refolding. This is in contrast with the conclusions of Tandon and Horowitz (1987), who found that rhodanese folding was promoted by several surfactants, but only above their CMC values.

Zardeneta and Horowitz (1992) have observed that binding to certain micelle systems slows rhodanese refolding but increases final recovery. The parallel to slowed refolding by promoters (Fig. 7A) is evident but clearly the mechanism is different.

Conclusions

Refolding CAII at high protein concentration produces a much higher yield than expected by extrapolation of earlier studies. This illustrates the risks involved in extrapolation when the reaction mechanism is complex. Our overall findings are in general agreement with the reaction scheme of Cleland et al. (1992), except that, under our conditions, turbidity decreases monotonically from a very early maximum, indicating partial reversibility of aggregate formation. About half of the 18 surfactants examined showed enhancement of CAII renaturation at 2.5 h. Several of these, including *n*-hexanol and *n*-pentanol, were effective promoters over a broad concentration range. Two of the surfactants decreased the renaturation rate at low protein concentration while increasing yield at high protein concentration. We repeat the above caution about extrapolations. Two effective promoters demonstrated no ability to dissolve aggregates. Moreover, if these promoters are not present at the initiation of refolding, they are ineffective. Therefore, these promoters do not act by dissolving aggregates but by suppressing initial aggregate formation. Micelle formation is not obligatory for promoter activity, inasmuch as half of the effective promoters do not form micelles. All of the candidates for promoter activity are surface active, yet only half of them were successful promoters. This implies a discrimination at the level of folding intermediate I_1, or its oligomers, with the result that some of the candidates form associated species that discourage early aggregate formation, whereas others do not. The apparent stoichiometry of successful promoters suggests interaction at the oligomer level of folding intermediates, but we must remember our own caution about rushing to interpret complex reactions. Further investigation is needed to explore the mechanism of these promoters and to determine whether they can enhance renaturation in other protein refolding systems.

Materials and methods

Materials

Bovine CAII (MW = 29,000, pI = 5.9), purified by electrophoresis, was purchased from Sigma Chemical Co., St. Louis, Missouri. The isozyme distribution was checked by IEF in our laboratory. Octyl glucoside, CHAPS, polyethylene glycol (mean MW, 8000), *p*-nitrophenyl acetate, Tris(hydroxymethyl)aminomethane (Trizma base, mol. biol. grade), and Na_2EDTA were also obtained from Sigma Chemical Co. Ultrapure GdmCl was the product of Schwartz/Mann Biotech, Cleveland, Ohio. The aliphatic alcohols (C_3–C_8), cyclohexanol, cyclohexyl ethanol, cycloheptanone, benzyl alcohol, and polyvinyl pyrrolidone (mean MW, 10,000) were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Mega-9 and Triton X-100 were obtained from Boehringer-Mannheim Corp., Indianapolis, Indiana. The detergent octaethylene glycol monolauryl ether ($C_{12}E_8$) was obtained from Calbiochem Inc., La Jolla, California. Zonyl FS-300, a water-soluble fluorocarbon surfactant, is a product of E.I. du Pont Corp., Wilmington, Delaware. *N*-octyl pyrrolidone was obtained from ISP Technologies(GAF), Wayne, New Jersey. Acetonitrile (HPLC grade) was a product of Fisher Scientific, Pittsburgh, Pennsylvania. All solutions were prepared with Milli Q water, and buffers were filtered through a 0.45-μ membrane before use.

Methods

Protein concentration

The concentration of native bovine CAII in 50 mM Tris-sulfate (pH 7.5) was determined by its absorbance at 280 nm with an extinction coefficient of 1.83 (mg/mL protein) cm^{-1} (Wong & Tanford, 1973). The extinction coefficient of denatured bovine CAII in 5.0 M GdmCl was determined experimentally to be 1.67 (mg/mL protein) cm^{-1} at 280 nm. For all the measurements, the denatured protein was prepared by mixing higher concentration solutions of native protein (in buffer) and GdmCl together, and allowing at least 12 h for denaturation, but not more than 24 h in the resulting 5.0 M GdmCl. GdmCl concentrations were determined refractometrically (Nozaki, 1972).

Refolding and esterase activity

The refolding was carried out by rapid dilution of denatured CAII in 5.00 M GdmCl to 1.00 M (or more dilute) GdmCl and the desired protein concentration, with a dilution buffer composed of 50 mM Tris-sulfate, pH 7.5. For testing the effects of additives, various surfactants were added to the dilution buffer. All the refolding operations and activity assays were carried out at 20.0 ± 0.3 °C. The activity assay was employed with *p*-nitrophenyl acetate (pNPA) as substrate (Pocker & Stone, 1967).

The enzymatic activity was measured after various refolding times by dilution of the assay sample 10-fold or 100-fold with 50 mM Tris sulfate, pH 7.50 buffer before adding 1/10 volume pNPA dissolved in acetonitrile to produce an initial concentration of 5×10^{-5} M pNPA. The formation of the product *p*-nitrophenolate was measured at 400 nm for 2 min with a Cary 210 spectrophotometer. The percent activity recovery was found by comparing with the activity of the native protein in the same concentration of GdmCl in the Tris-sulfate buffer and any additive used. A correction is made for the nonenzyme-catalyzed hydrolysis of pNPA. Because aggregation sometimes occurs in the activity assay, a 2-min measurement of turbidity is also carried out at 400 nm, with the diluted protein solution having the same composition as in the assay but lacking substrate. The rate of apparent absorbance (turbidity) increase in the absence of substrate is subtracted from that in the presence of substrate to give a corrected measure of enzyme activity. Larger turbidity corrections are seen at early times and higher protein concentrations. At 4 mg CAII/mL, the turbidity contribution at 5 min was 9–14%; at 30 min the turbidity contribution was 4–7% of the apparent rate.

To test the effects of a large number of surfactant additives, each over a substantial concentration range, we compared the activity yields at 150 min refolding for CAII at 4.0 mg/mL and GdmCl at 1.00 M. For each additive, refolding was carried out over a series of concentrations. Duplicate determinations of activity were made (at 150 min and 153 min). A separate control containing no additive was included in each set of measurements. Enzyme activity assays for Figures 6 and 7 were carried out with 4 mM EDTA in the assay solution, to suppress refolding during the assay. EDTA does not inactivate native CAII. The assays for Figures 1, 3, 4, and 5 were carried out without EDTA as a quench in the assay solution. For rapidly regenerating solutions, this leads to somewhat higher estimates of activity than what is found with EDTA in the assay solutions. However, we found that, for activity measurements at 150 min, when the activity changes very slowly with time, the same concentration of enzyme is found with or without EDTA in the assay solution.

The presence of EDTA (5 mM) in the refolding solvent, as specified by Cleland and Wang (1990a, 1990b), completely suppresses formation of esterase activity. It appears likely that this specification was a simple error of fact that eluded proofreading. This is the more plausible because these authors also indicate that EDTA was used in the assay dilution buffer (which is sensible because it stops continuing formation of active enzyme in the assay solution). Also, none of the earlier studies of CAII refolding employed EDTA in the refolding solvent, but where comparisons are possible, there appears to be consistency between Cleland and Wang (1990a, 1990b), Cleland et al. (1992), and earlier investigations. We therefore assume, as a working premise, that Cleland and Wang did not employ EDTA in their refolding solutions that regenerated CAII activity.

Turbidity measurement in refolding solvent

The denatured protein in 5.00 M GdmCl was diluted rapidly to 1.00 M GdmCl with 50 mM Tris-sulfate buffer, pH 7.5, with or without additives. The turbidity of the solutions was measured as apparent absorbance at 330 nm after 10 s manual (end-over-end) mixing in cells of 10.0-mm pathlength, and thereafter turbidity measurements were made at frequent intervals, each measurement preceded by manual mixing. Replicate experiments showed substantial variability in the initial measurements, presumably due to variability in the manual mixing. The data shown in Figure 3 are representative data.

CMC determinations

Using the fluorescence titration method of Horowitz (1977), CMC determinations were carried out for CHAPS and for $C_{12}E_8$ in 1.00 M GdmCl (containing 50 mM Tris sulfate, pH 7.5) at 20 °C. We also determined the CMC in the buffer alone. For CHAPS, CMC (in GdmCl) = 10 mM, CMC (in buffer) = 4 mM. For $C_{12}E_8$, CMC (in GdmCl) = 0.17 mM, CMC (in buffer) = 0.07 mM.

Surface tension

Employing a Du Nouy ring apparatus, we measured the surface tensions of a range of concentrations of ethanol, *n*-butanol, *n*-hexanol, and *n*-octanol in 50 mM Tris sulfate buffer, pH 7.5, 22–23 °C. A similar set of measurements was made with 1.00 M GdmCl containing the same buffer components. The GdmCl solution and the aqueous buffer showed the same surface tension within 1%; plots of surface tension versus [alkanol] were identical within experimental error (est. ±1 dyne/cm) for solutions with and without GdmCl, and the buffer-only solutions agree well with published values found in pure water (Posner et al., 1952).

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