Polyethylene Glycol Enhanced Refolding of Bovine Carbonic Anhydrase B

REACTION STOICHIOMETRY AND REFOLDING MODEL*

(Received for publication, January 16, 1992)

Jeffrey L. Cleland‡, Chester Hedgepeth, and Daniel I. C. Wang‡
From the Biotechnology Process Engineering Center, Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. ‡Chemical Research and Development, Genentech Inc., South San Francisco, California 94080, and the ‡Department of Biology, University of Maryland Baltimore County, Baltimore, Maryland 21228

Polyethylene glycol (PEG) inhibited aggregation during refolding of bovine carbonic anhydrase B (CAB) through the formation of a nonassociating PEG-intermediate complex. Stoichiometric concentrations of PEG were required for complete recovery of active protein during refolding at aggregating conditions. For example, a PEG (Mn = 3550) to CAB molar ratio ([PEG]/[CAB]) of 2 was sufficient to inhibit aggregation during refolding at 1.0 mg/ml (33.5 μM) protein and 0.5 M guanidine hydrochloride. In addition, the PEG concentration required for enhancement was dependent upon the molecular weight and only molecular weights between 1000 and 8000 were effective in inhibiting aggregation. In the presence of PEG, the rate of refolding was the same as that observed for refolding without the formation of associated species. Refolding in the presence of PEG resulted in the rapid formation of a PEG complex with the molten globule first intermediate, and this PEG-intermediate complex did not aggregate. The CAB refolding kinetics in the presence of PEG were determined and used to develop a model of the PEG enhanced refolding pathway. The mathematical model was validated by independent activity measurements of CAB refolding. This model predicted that PEG enhanced refolding of CAB occurred by a specific interaction of PEG with the molten globule first intermediate to form a nonassociating complex which continued to fold at the same rate as the first intermediate. The predicted pathway and binding properties of PEG indicate that PEG enhanced refolding may be analogous to chaperonin mediated protein folding.

Previous studies of bovine carbonic anhydrase B (CAB)‡

* This work was supported by National Science Foundation Grant CDR-88-09014. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all correspondence and reprint requests should be addressed. Tel.: 617-253-2126. Fax: 617-253-2400.

The abbreviations used are: CAB, bovine carbonic anhydrase B; GdnHCl, guanidine hydrochloride; PEG, polyethylene glycol; pNPA, p-nitrophenol acetate; pNP, p-nitrophenol; HPLC, high performance liquid chromatography; [CAB]f, final protein concentration after dilution (mg/ml or μM); [D], dimer concentration (μM); [GdnHCl]f, final GdnHCl concentration after dilution (M); [I], concentration of first intermediate in CAB refolding (μM); [I]1, CAB first intermediate concentration at given time, t; [I]-P, CAB first intermediate-PEG complex concentration (μM); [IL], concentration of second intermediate in CAB refolding (μM); [UL], CAB second intermediate concentration at given time, t; kapp, apparent rate constant for formation of second intermediate in CAB refolding pathway (1/min); kassoc, association rate constant for dimer formation (μM-1 min-1); kdis, dissociation rate constant for dimer (1/min); k1, rate constant for formation of first intermediate in CAB refolding (1/s); k2, rate constant for formation of second intermediate in CAB refolding pathway (1/min); k3, rate constant for forming to native state (1/min); kassoc, association rate constant for first intermediate-PEG complex; kdis, dissociation rate constant for first intermediate-PEG complex; kassoc, association rate constant for trimer formation (μM-1 min-1); k5, dissociation rate constant for trimer (1/min); Keq, equilibrium constant for dimer in the presence of PEG (μM-1); [N], native protein concentration (μM); [N0], native protein concentration at given time, t; [P], PEG concentration (mM); [PEG]f, final concentration of PEG after dilution (g/liter); R1/2, initial rate of refolding (1/min); t, time after dilution to final conditions (min or s); t1/2, half-time for refolding reaction step (min or s); [U], unfolded protein concentration (μM).
glycol (Mₚ = 300, 400, 1000, 3350, and 8000), and p-nitrophenyl acetate (pNPA) were purchased from Sigma as molecular biology grade reagents. The purity of each lot of CAB (pI = 5.9) was confirmed by gel electrophoresis analysis with silver staining. HPLC grade acetonitrile was obtained from J. T. Baker Chemical Co. Distilled deionized water was from MilliQ water purification system (Millipore) was used to prepare all buffers and samples.

Methods

Protein Concentration—To determine the protein concentration of native CAB in 50 mM Tris sulfate, 5 mM EDTA, at pH 7.5, absorbance at 280 nm was measured as described previously (Cleland and Wang, 1992). An extinction coefficient of 1.83 (mg/ml protein)⁻¹ cm⁻¹ was used to calculate the protein concentration from the absorbance at 280 nm with a molecular weight of 30,000 (Wong and Tanford, 1973). Protein denatured in GdnHCl was measured with a colorimetric dye binding assay (Bio-Rad) as described previously (Cleland and Wang, 1990a).

Esterase Activity—The activity of each protein solution was determined by using the esterase assay activity as described by Pocker and Stone (Pocker and Stone, 1967; Cleland and Wang, 1990a). Briefly, CAB in 5 M GdnHCl was rapidly diluted to the desired final GdnHCl and protein concentrations. The refolding solution was then analyzed for enzymatic activity at various times after dilution. Prior to addition of substrate, pNPA, each assay sample was diluted 10-fold by 50 mM Tris sulfate, 5 mM EDTA, pH 7.5. The formation of the product from the enzyme action, p-nitrophenol (pNP), and decrease in the substrate, pNPA, were measured by absorbance at 348 nm and 400 nm, respectively, as described previously (Cleland and Wang, 1990a). The esterase hydrolysis rate constant of the native protein with or without PEG at the same concentration in the dilution buffer (50 mM Tris sulfate, 5 mM EDTA, pH 7.5) was used to calculate the activity of the protein in each refolding experiment. PEG was not observed to increase the hydrolysis rate of pNPA in the presence of native CAB.

HPLC Analysis—Size exclusion chromatography was performed with a Phenomenex Pak 3000 SW column (Waters Associates), as described previously (Cleland and Wang, 1991). Refolding of denatured CAB in 5 M GdnHCl was achieved by rapid dilution to different GdnHCl concentrations and 1.0 mg/ml protein with 30 g/liter PEG (Mₚ = 8000). To assess the effect of PEG on the equilibrium distribution of multimers, different final PEG (Mₚ = 8000) concentrations were used at a final protein concentration of 1.0 mg/ml in 2.0 M GdnHCl. After equilibration, the sample was applied to the size exclusion column at a flow rate of 1.0 ml/min to facilitate rapid separation.

By using a previously developed method, the concentration of each species was calculated from the area of the absorbance (280 nm) peak (Cleland and Wang, 1991). The elution properties of the column in 2 M GdnHCl and various PEG concentrations was determined by using low concentrations of CAB (<10 μM) where the protein does not associate (Cleland and Wang, 1991). In addition, bovine serum albumin was also utilized to measure the changes in the elution time which result from the presence of PEG in the elution buffer.

Absorbance Measurements of Refolding Kinetics—To measure the rate of formation of the second intermediate and native protein, the change in the aromatic amino acid residues was measured at 280 nm as described previously (Cleland and Wang, 1992; Semisotnov et al., 1990; Wong and Tanford, 1973). In summary, denatured CAB in 5 M GdnHCl was rapidly diluted to 1.0 M GdnHCl and different protein concentrations with and without PEG (Mₚ = 3350). After dilution, the solution absorbance at 280 nm was measured immediately (dead time ≈ 0 s) at 1 s intervals for 700 s. The observed refolding rates for the two phases were calculated from the slopes of the lines generated from a least squares fit of a semilogarithmic plot of the data (Wong and Tanford, 1973). The change in absorbance was measured in duplicate for each final condition. Each experiment at the same conditions resulted in the nearly identical rate constant values (±2%), and the rate constants were accurately (±10%) fit to the absorbance data.

Results and Discussion

To determine the effect of PEG on CAB refolding pathway, experiments were first performed at final conditions which resulted in the transient association of the first intermediate (1.0 M GdnHCl and 0.50 mg/ml (16.7 μM) CAB) (Cleland and Wang, 1992). In addition, the relationship between the PEG concentration and the rate of refolding was determined to provide insight into the specific stoichiometry and mechanism of PEG interaction. The stoichiometry between PEG and CAB was also assessed at aggregating conditions (0.5 M GdnHCl and 1.0 mg/ml (33.3 μM) CAB) (Cleland and Wang, 1990a). To further characterize the interaction of PEG with the protein, refolding was attempted at these aggregating conditions with PEG at different molecular weights. These studies provided additional information on the mechanism of PEG-enhanced refolding.

Stoichiometry of PEG Enhancement and Apparent Refolding Rates—Previous studies on PEG enhanced refolding of CAB indicated that the molar ratio of PEG at the protein surface to partially folded inactive protein was 2 to 1 (Cleland and Wang, 1990b). Therefore, the concentration of PEG (3 g/liter, 885 μM) used in the previous refolding experiments was greater than that required to facilitate refolding of the denatured CAB which was refolded at a concentration of 0.50 mg/ml (16.7 μM). To determine a more detailed relationship between PEG concentration and enhancement, refolding experiments were performed at several molar ratios of PEG (Mₚ = 3350) to protein. Refolding of CAB in 5 M GdnHCl was performed by rapidly diluting denatured CAB in 5 M GdnHCl to 0.50 mg/ml CAB and 1.0 M GdnHCl with PEG (Mₚ = 3350) in the dilution buffer. The initial rate of recovery of active protein, Rₑf₁, was calculated from the slope of the activity as a function of time at each molar ratio as described previously (Cleland and Wang, 1990b). As shown in Fig. 1, the maximum observed rate enhancement occurred at a molar ratio ([PEG]/[CAB]) between 2 and 3 and resulted in a 3-fold increase in the apparent initial rate of refolding. At the optimal ratio, the PEG ([PEG]/[CAB]) = 3350) concentration was between 0.15 and 0.20 g/liter.

By using the assumption that PEG only interacts with the first intermediate in the refolding pathway, the increase in the apparent refolding rate from the first to second intermediate was calculated. The apparent rate constant for formation of the second intermediate, kₑf₂, was then determined for each case as described previously (Cleland and Wang, 1992). The maximum apparent rate constant, kₑf₂, was 1.08 min⁻¹.
which is comparable to the actual second intermediate rate constant ($k_3 = 1.386 \text{ min}^{-1}$) determined for refolding at low protein concentrations (<10 μM) and 1.0 M GdnHCl where the first intermediate does not form a transient dimer species (Cleland and Wang, 1992). In addition, HPLC size exclusion analysis of the PEG enhanced refolding at 0.50 mg/ml (16.7 μM) CAB, and 1.0 M GdnHCl revealed that the transient dimer did not form during the refolding (data not shown). Therefore, the PEG must be preventing the association of the first intermediate for refolding at the conditions where the formation of a transient dimer was previously observed.

Since PEG was observed to prevent the formation of a transient dimer species at stoichiometric concentrations, a similar relationship should also exist for the reduced formation of dimers and trimers during refolding at aggregating conditions. To test this hypothesis, refolding at aggregating conditions of 1.0 mg/ml CAB and 0.50 M GdnHCl was attempted at molar ratios of PEG ($M_f = 3350$) to CAB ranging from 1 to 9. CAB in 5 M GdnHCl was rapidly diluted to 1.0 mg/ml (33.3 μM) protein and 0.50 M GdnHCl with PEG in the dilution buffer, and the concentration of active protein was then measured as shown in Fig. 2. The protein precipitated out of solution without PEG in the dilution buffer and resulted in the recovery of only 90% active protein. A one to one molar ratio of PEG to CAB provided only slightly higher recovery of active protein (35%), and precipitation of inactive protein was again observed. However, molar ratios ([PEG]/[CAB]) of 2, 3, and 9 resulted in complete recovery of active protein after 1 h without the formation of precipitates and multimers. Therefore, the minimum concentration of PEG ($M_f = 3350$) required to achieve complete recovery at these aggregating conditions was 0.35 g/liter. These results along with the refolding rate enhancement (Fig. 1) have indicated that a minimum molar ratio of PEG ($M_f = 3350$) to CAB of 2 to 1 was required to prevent the formation of the multimers and, thereby, achieve increased recovery of active protein.

**Effect of PEG Molecular Weight and Concentration**—The previous studies showed a distinct stoichiometric relationship between PEG ($M_f = 3350$) and the prevention of first intermediate association. To develop a further understanding of this relationship, refolding of CAB was performed by using PEG at different molecular weights. Denatured CAB in 5 M GdnHCl was refolded by rapid dilution to 1.0 mg/ml (33.3 μM) and 0.50 M GdnHCl with PEG in the dilution buffer at different molar ratios. Each solution was then allowed to equilibrate for 1 h. After equilibration, the active protein concentration of each solution was measured as shown in Fig. 3a. With $M_f = 8000$ PEG in the dilution buffer, complete recovery of active protein was achieved for a narrow operating range of molar ratios (2 to 10). At a lower PEG molecular weight of 3350, the range of molar ratios required for complete recovery was somewhat broader (2 to 20). This trend continued for the $M_f = 1000$ PEG which had a broader operating range (10 to 40) and required a higher minimum molar ratio (10) for complete recovery. When smaller molecular weight PEG (200 and 600) was used in the dilution buffer, the protein did not completely recover activity for any of the molar ratios tested. In addition, when high molecular weight PEG ($M_f = 20,000$) was used in the dilution buffer, the recovery of active protein decreased and the extent of aggregation increased for all molar ratios ([PEG]/[CAB]) = 0.1 to 10; data not shown.)
These results corroborate the similar phenomenon of PEG adsorption to silica surfaces (Cher et al., 1988, 1989). For PEG adsorption on silica, the polymer will loop down and form a flat interface with the surface having the tail pointing into the solvent. The degree of surface packing has been shown to be dependent on the polymer molecular weight. In other words, as the chain length increases, the amount of polymer in contact with the surface increases resulting in less polymer required to cover the surface. After complete surface packing is achieved, addition of polymer results in a stacking effect where the segment length of the polymer in contact with the surface decreases and the number of polymers at the interface increases. For the low molecular weight polymers (200 and 600), a stacking of the polymers at the surface may occur such that the polymer stacks on top of itself instead of spreading across the surface. This polymer adsorption theory would explain the molar ratios and stoichiometry observed in refolding of CAB with different PEG molecular weights. At high molecular weights (1000 to 8000), PEG may follow a similar adsorption pattern of packing on the surface of CAB. Lower molecular weight PEG (200 and 600) may not provide protection against aggregation since the polymer will not spread across the protein surface to cover the hydrophobic sections which are the likely cause of aggregation. A very large polymer such as the $M = 20,000$ PEG could adsorb to more than one protein molecule and cause an increase in the effective concentration of the protein and a subsequent increase in aggregation. Thus, refolding with PEG at different molecular weights would be predicted by polymer adsorption theory to occur with a stoichiometry similar to that shown in Fig. 3a.

The refolding experiments involving PEG at different molecular weights showed the specific stoichiometry of interaction between PEG and CAB. However, to uncouple the effect of the mass concentration and molecular weight, these results were replotted as a function of the mass concentration of PEG. The data from Fig. 3a were therefore replotted as the final recovery of active protein at each mass concentration of PEG (Fig. 3b). The combined results of experiments using PEG ranging from $M = 200$ to 20,000 revealed that mass concentrations between approximately 0.20 g/liter and 3 g/liter provided complete recovery of active protein under these final conditions. The data below the optimum mass concentration range for complete recovery of active protein as shown in Fig. 3b represented the use of PEG molecular weights of 200, 600, and 20,000 where complete recovery was not achieved. The observed mass concentration effect may be explained by adsorption to the protein at low polymer concentrations and exclusion of the polymer from the protein surface at high polymer concentrations. A similar concentration effect was observed previously for PEG interaction with native proteins (Arakawa and Timasheff, 1985).

These refolding and aggregation studies have shown that the association of the first intermediate can be prevented through the application of specific molar ratios of PEG to CAB which are dependent upon the PEG molecular weight. PEG will interact with the first intermediate and prevent its association, but the distinct mechanism of this interaction cannot be clearly elucidated from these experiments. PEG may alter the association process by dissociating the dimer through an increase in the dissociation rate, binding to the first intermediate, or formation of a new intermedaete species which does not associate. To discriminate between each of these mechanisms, the effect of PEG on the equilibrium association of the first intermediate must be determined.

**PEG Effect on Equilibrium Association of CAB—**If PEG results in the formation of a new stable intermediate state or the dissociation of the dimer species, the equilibrium distribution of associated protein will be different. This effect was initially observed in previous studies which revealed a shift in the equilibrium distribution toward the monomer in the presence of PEG for refolding in 2 M GdnHCl (Cleland and Wang, 1990b). First of all, if a new stable intermediate state is formed in the presence of PEG, the association dependence on the final GdnHCl concentration should be altered since a higher GdnHCl concentration would be required to desalubize the protein. On the other hand, if PEG only causes a dissociation of the multimer, a decrease in protein association should occur with increasing PEG concentration. Therefore, equilibrium protein association experiments were performed for different final conditions to elucidate the different phenomena.

To determine if PEG causes the formation of a new stable intermediate species, equilibrium refolding experiments were performed at 1.0 mg/ml CAB and final GdnHCl concentrations ranging from 1.0 to 2.5 M in 30 g/liter PEG ($M = 8000$) as performed previously without PEG (Cleland and Wang, 1991). Each of these solutions was allowed to equilibrate for 2-8 h. After equilibration, the concentration of each species was determined by HPLC size exclusion analysis and plotted as a function of the final GdnHCl concentration (Fig. 4). As shown in Fig. 4, the association of CAB had the same dependence on the final GdnHCl as was observed without PEG (Cleland and Wang, 1991). The association was the greatest for final GdnHCl concentrations ranging from 1.8 to 2.2 M GdnHCl. Since PEG did not shift the dependence on the final GdnHCl concentration and the first intermediate at 2.0 M associated, the formation of a new stable intermediate state was not observed. However, the extent of association of the first intermediate at 2.0 M GdnHCl was greatly reduced by the presence of PEG indicating a possible dissociation mechanism. The trimere species was not observed to form at any final GdnHCl concentration at these conditions. PEG must then act by shifting the equilibrium association of the first intermediate at 2 M GdnHCl to the monomer.

To determine the relationship between the equilibrium distribution and the PEG concentration, equilibrium association studies were performed at 2.0 M GdnHCl and 1.0 mg/ml CAB (33.3 $\mu$M) with several different PEG ($M = 8000$)

**Fig. 4. Equilibrium association as a function of GdnHCl concentration in 30 g/liter PEG ($M = 8000$).** CAB in 5 M GdnHCl (GuHCl) was diluted to 2 M GdnHCl and 1.0 mg/ml protein with PEG in the dilution buffer. The concentrations of monomer (•) and dimer [() were determined by HPLC size exclusion analysis as described under “Experimental Procedures.” The trimer species was not observed under these conditions.
concentrations. Denatured CAB in 5 M GdnHCl was rapidly
diluted to the final conditions with concentrated PEG solutions
to yield final PEG concentrations from 1 to 60 g/liter. Higher
concentrations of PEG were not attempted since precipitation was observed at PEG concentrations greater
than 50 g/liter. The solutions were allowed to equilibrate for
3-6 h. After equilibration, the samples were analyzed by
HPLC size exclusion, and the PEG and GdnHCl concentrations
in the elution buffer were the same as the sample (see
"Experimental Procedures"). The distribution of monomer,
dimer, and trimer for each PEG concentration is shown in
Fig. 5a. These results did not indicate the presence of a trimer
species as observed by quasi-elastic light scattering (QLS)
analysis since the time period for HPLC analysis was longer
than the equilibrium between the dimer and trimer species as
discussed previously (Cleland and Wang, 1991). At PEG
concentrations less than 30 g/liter, the distribution shifts to
the monomer. PEG concentrations greater than 30 g/liter resulted in a shift in the distribution to the dimer species.
These results are explained by the two mechanisms of PEG
interaction. At low concentrations, PEG will interact with the
first intermediate and prevent its association. PEG will be
excluded from the protein surface (preferential hydration) at
high concentrations, and this exclusion will result in a return
to association conditions.

To observe the apparent shift in the equilibrium distribution,
the equilibrium constant for dimer formation was cal-
culated and plotted as a function of the PEG concentration as shown in Fig. 5b. For this calculation, the overall monomer
concentration was used instead of the first intermediate
centration since the first intermediate and PEG have been
shown to bind forming a PEG-intermediate complex (Cleland
and Randolph, 1992). The apparent equilibrium constant, $K'_d$, was therefore defined to be:

$$K'_d = [D]/[M]^2$$

(1)

where $[M]$ and $[D]$ are the concentrations of monomer and
dimer as measured by HPLC size exclusion analysis (Fig. 5a).
The equilibrium constant decreased dramatically with in-
creasing PEG concentrations (Fig. 5b). This relationship con-
firmated the relationship between low PEG concentrations and
reduced association as observed for the kinetic studies on
refolding with PEG (Fig. 3b).

Overall, equilibrium protein association studies in the pres-
ence of PEG have shown that the association of the first
intermediate can be avoided at low PEG concentrations (<30
g/liter). PEG prevented the association of the first interme-
tiate, and the relationship between the final GdnHCl concen-
tration and the extent of association were not altered by PEG.
Therefore, PEG did not form a new intermediate species which
was more stable in GdnHCl. These studies have shown that
PEG will reduce association, but the mechanism of the
reduction in association has not been demonstrated. It is
conceivable that PEG will either cause the dissociation of the
dimer species or prevent the association by binding to the
protein surface.

**Proposed Pathway for PEG Enhanced Refolding of CAB**—
To develop a pathway for PEG enhanced refolding of CAB,
the results of several different analyses must be combined to
provide a complete and accurate model. First of all, kinetic
studies on the refolding and aggregation of CAB in the pres-
ence of PEG demonstrated that low molar ratios of PEG to
CAB resulted in an increased recovery of active protein and
a decrease in multimer formation (Figs. 2 and 3). These
studies have also shown that low concentrations of PEG
prevented the formation of transient dimer species during
refolding at high protein concentrations (>10 μM) and 1.0 M
GdnHCl (Fig. 1). The ability of PEG to prevent association of
the first intermediate was further confirmed by equilibrium
studies at high protein concentrations (>10 μM) and 2.0 M
GdnHCl. In these equilibrium studies, PEG ($M_f = 8000$)
at low concentrations (<30 g/liter) greatly reduced the associa-
tion of the first intermediate (Fig. 5). Therefore, the pathway
for PEG enhanced refolding would include a reduction in
association of the first intermediate at equilibrium and during
refolding. The reduction could occur by a decrease in the
dimer association rate or an increase in the dimer dissociation
rate. Alternatively, the first intermediate could be shifted
from the normal refolding pathway to a stable PEG-first
intermediate complex which does not associate.

To determine the mechanism of this reduced association,
the interaction of PEG with the first intermediate was deter-
mined. Equilibrium binding experiments revealed that PEG
bound to the first intermediate in the refolding pathway, but
PEG did not bind to the second intermediate or native protein
(Cleland and Randolph, 1992). In addition, it was shown that
the first intermediate had one primary binding site for PEG
($M_f = 8000$) and several minor binding sites (Cleland
and Randolph, 1992). The binding of PEG to the first intermediate

![Graph](image1.png)

**Fig. 5.** PEG concentration effect on equilibrium association of first intermediate. Equilibrium refolding experiments were performed by rapid dilution from 5 M GdnHCl to 2 M GdnHCl and 1.0 mg/ml (33.3 μM) CAB with different concentrations of PEG ($M_f = 8000$). a, the concentration of monomer ($\Theta$), dimer ($\mathbb{D}$), and trimer ($\mathbb{D}^+$) was determined by HPLC size exclusion analysis. b, the apparent equilibrium constant for dimer formation, $K'_d$, was calculated from the multimer concentration data in graph a by using Equation 1.
PEG Enhanced Refolding of CAB

resulted in changes in the properties of the intermediate. Furthermore, these studies indicated that the surface properties of the first intermediate were significantly altered as a result of bound PEG (Cleland and Randolph, 1992). Therefore, the proposed model for refolding should include the binding of PEG to the surface of the first intermediate with concomitant changes in its surface properties such that it cannot associate to form dimers or trimers.

The rate constant for the formation of second intermediate was measured by the absorbance analysis technique described previously to determine if the refolding rate from the first intermediate to the second intermediate increased in the presence of PEG (Cleland and Wang, 1992; Semisotnov et al., 1990; Wong and Tanford, 1973). This analysis was completely independent of the previous activity studies. To determine the rate constant for second intermediate formation, refolding from 5 M GdnHCl to 1.0 M GdnHCl and 0.50 mg/ml (16.7 μM) protein was performed with PEG (Mₚ = 3350) in the dilution buffer at a final concentration of 0.17 g/liter ([CAB]/[PEG] = 3). After dilution, the refolding was measured by the change in absorbance at 280 nm as shown in Fig. 6. As observed for the previous absorbance kinetic studies, the change in absorbance could be modelled by two exponential phases (Cleland and Wang, 1992; Semisotnov et al., 1990; Wong and Tanford, 1973). The rate constant for the second exponential was 7.76 × 10⁻² min⁻¹ (tᵣ₂ = 531 s) which is within experimental error of the previously measured rate constant (kₛ = 7.24 × 10⁻² min⁻¹, Cleland and Wang, 1992) and has been previously shown to be the rate constant for native protein formation (Semisotnov et al., 1990). The rate constant for the first exponential which is the rate constant for the formation of the second intermediate was the same as that determined for refolding at the nonassociating condition of low protein concentration (<10 μM) and 1.0 M GdnHCl (kₛ = 1.39 min⁻¹, tᵣ₁ = 30 s; Cleland and Wang, 1992). Refolding in the presence of PEG must therefore proceed through a nonassociating first intermediate structure which folds to the second intermediate at the same rate.

To depict each of the observed phenomena of PEG enhanced refolding of CAB, the pathway shown in Fig. 7 was developed. When diluted to low GdnHCl concentrations (≤2.0 M), the unfolded protein, U, in 5 M GdnHCl will rapidly form the first intermediate, I₁. The first intermediate may then proceed through three different pathways. Since PEG has been observed to bind to the first intermediate and prevent the association of this intermediate, a PEG-first intermediate complex, I₁-P, which does not associate must be formed during the refolding process. Also, the surface properties of the first intermediate were altered in the presence of PEG as observed by fluorescence and ESR studies (Cleland and Randolph, 1992). Therefore, the PEG-intermediate complex is distinct from the first intermediate in the absence of PEG. Without PEG in the refolding buffer, the first intermediate may associate to form the dimer, D, or refold to the second intermediate, I₂. Since the rate of refolding to the second intermediate with PEG is the same as that observed for refolding at low protein concentrations (<10 μM) in 1.0 M GdnHCl, the folding of the PEG-first intermediate complex to the second intermediate has the same refolding rate constant (kₛ). The number of PEG molecules required to form the PEG-intermediate complex has been shown to be dependent upon the molecular weight of the polymer. Unlike the association reaction, the molar ratio of PEG to protein ([PEG]/[CAB]) required for enhancement was independent of the final GdnHCl concentration (Figs. 1 and 2). Since PEG was required in at least a 2-fold molar excess for each PEG molecular weight (Fig. 3), and the equilibrium constant for dimer formation was significantly reduced in the presence of low PEG concentrations (Fig. 5), the equilibrium between the first intermediate and the PEG-intermediate complex must be shifted toward the nonassociating complex. The rate at which the nonassociating complex forms may be expressed as the rate of association (kₛ > kₐ) since the dimer was not observed for refolding in PEG at conditions where the transient dimer formed (Fig. 1) or conditions where irreversible association occurred (Fig. 2). Overall, this pathway represents the rapid formation of the nonassociating species, I₁-P, followed by the folding of this species to the second intermediate at the same refolding rate as that observed in the absence of association.

Mathematical Model of Pathway and Refolding Rates—Kinetic and equilibrium refolding studies which were performed with PEG have shown that multimers will not form at aggregating conditions. Therefore, the rate of formation of the PEG-intermediate complex must be much greater than the rate of dimer formation for the final conditions studied (kₛ >> kₐ). The rate expression for the PEG-intermediate complex may then be written as:

$$\frac{d[I₆P]}{dt} = kₛ[I₁P] - kₐ[I₆P]$$

The first intermediate is formed very rapidly upon dilution of...
denatured CAB in 5 M GdnHCl (Semisotnov et al., 1990). In addition, the concentration of the first intermediate is quickly reduced in the presence of PEG to prevent the formation of dimers. The rate equation for first intermediate can then be written as:

\[
\frac{d[I_1]}{dt} = -k_o[I_1] - k_p[I_1] - k_{o2}[I_1]
\]  

(3)

Since the first intermediate rapidly achieves a steady state, the steady state approximation \(d[I_1]/dt = 0\) can be applied to obtain a relationship between the first intermediate and the PEG-intermediate complex:

\[
k_o[I_1] - k_p[I_1] = -k_{o2}[I_1]
\]  

(4)

Substituting Equation 4 into Equation 2 yields:

\[
\frac{d[I_1]}{dt} = -k_o[I_1] + [I_1][P]
\]  

(5)

The first intermediate will quickly proceed to form the PEG-first intermediate complex as shown in Fig. 7. The initial concentration of this complex would then be equivalent to the total protein concentration, \([\text{CAB}]_0\). The concentration of the complex must then be much greater than the concentration of the first intermediate \((I_1[P] \gg [I_1])\) at steady state and, thus, Equation 5 reduces to:

\[
\frac{d[I_1]}{dt} = -k_o[I_1] - [I_1][P]
\]  

(6)

The concentration profile for the intermediate complex can then be written as:

\[
[I_1][P] = [\text{CAB}][\exp(-k_o t)]
\]  

(7)

Since the complex forms very rapidly, the concentration of the first intermediate which folds to form the second intermediate is negligible. The rate expression for the second intermediate could then be expressed as:

\[
\frac{d[I_2]}{dt} = k_o[I_1][P] - k_{o2}[I_2]
\]  

(8)

and the rate expression for the formation of the native protein is described by:

\[
\frac{d[N]}{dt} = k_o[I_2]
\]  

(9)

By using Equations 7, 8, and 9, the concentration profiles for the second intermediate and the native protein were determined to be:

\[
[I_2] = \left(\frac{k_o[\text{CAB}]}{k_o - k_{o2}}\right)\left\{[I_1]\exp(-k_o t) - \exp(-k_{o2} t)\right\}
\]  

(10)

\[
[N] = [\text{CAB}]_0\left\{\left(\frac{k_o}{k_o - k_{o2}}\right)\exp(-k_o t) - \left(\frac{k_{o2}}{k_o - k_{o2}}\right)\exp(-k_{o2} t) + 1\right\}
\]  

(11)

Equations 7, 10, and 11 completely describe the refolding pathway shown in Fig. 7 and can be applied to all cases where the maximum PEG enhancement has been observed. Thus, this model can be applied to conditions where PEG prevents the formation of an associated species. If an associated species does form, the rates of both the association and the PEG-intermediate complex formation must be determined to derive an accurate model.

The concentration of each protein species can be calculated based on this mathematical model for refolding with PEG.
for conditions where PEG prevents the formation of multimers.

CONCLUSIONS

Polyethylene glycol enhanced refolding of CAB has been shown to occur through the formation of a nonassociating PEG-first intermediate complex. This polymer-protein complex has been previously characterized (Cleland and Randolph, 1992). The concentration of PEG required to form the complex and achieve an increase in the recovery of active protein at aggregation conditions is twice the total protein concentration for $M_r = 3350$ PEG. The stoichiometry required for the reaction is also dependent on the polymer molecular weight and only PEG with molecular weights of 1000 to 8000 yields complete recovery of active protein. In addition, the folding reaction involving the formation of the second intermediate occurs at the same rate for the PEG-intermediate complex. Therefore, PEG does not catalyze the refolding process, but it does prevent self-association of the first intermediate. The model pathway developed from these observations has been described mathematically. The mathematical model prediction of active protein concentration is in excellent agreement with the independently measured active protein concentration. Thus, the PEG enhanced refolding of CAB occurs through the formation of a nonassociating intermediate complex which increases the recovery of active protein at aggregating conditions, and the overall pathway as shown in Fig. 7 is a possible model for PEG enhanced refolding.

The PEG enhanced refolding pathway has many analogies to proposed chaperonin mediated folding pathways. Several researchers have observed that the chaperonin, GroEL, reversibly binds to molten globule folding intermediates (Martin et al., 1991; Landry and Giersch, 1991). However, unlike PEG, energy (triphosphate nucleotides) and the chaperonin, GroES, are required to release the molten globule from GroEL and allow the protein to finish folding (Martin et al., 1991; Hubbard and Sanders, 1991). GroEL has also been observed to prevent aggregation of partially folded proteins (Buchner et al., 1991). Therefore, both PEG and the chaperonin, GroEL, bind to partially folded structures and inhibit their aggregation without altering the rate of folding (Buchner et al., 1991). In addition, the off-pathway formation of a chaperonin-intermediate protein species has been proposed for chaperonin-mediated folding both in vivo and in vitro (Buchner et al., 1991; Hubbard and Sanders, 1991). Additional studies of the specific physicochemical interactions of PEG and chaperonins with folding intermediates should provide insight into these observed similarities.

REFERENCES

Cleland, J. L., and Wang, D. I. C. (1990b) Biotechnology 8, 1274-1276