Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 60-66

CYCLODEXTRINS AS PROTEIN FOLDING AIDS

N. Karuppiah and A. Sharma*

Department of Chemistry, Central Michigan University
Mt. Pleasant, MI 48859

Received March 20, 1995

Summary: Aggregation of proteins is a frequent occurence during their transition from random coil to native structure. The influence of cyclodextrins in the refolding of carbonic anhydrase under aggregating conditions was studied. Cyclodextrin prevented formation of protein aggregates during renaturation of carbonic anhydrase. In addition, over 90 % of active enzyme was recovered even at protein concentrations as high as 67 μM. The enhanced protein reactivation by cyclodextrins may be due to their ability to bind to hydrophobic sites in protein folding intermediate(s) followed by their subsequent removal as the protein refolds.

Over 30 years ago, it was shown that the three dimensional structure of a protein is determined by its amino acid sequence (1). Understanding how a linear protein chain folds into its unique three dimensional structure (native state) represents a major challenge. An understanding of this basic question has recently gained immense commercial importance in the production of recombinant human protein pharmaceuticals such as insulin,tissue plasminogen activator and human deoxyribonuclease (2). Many of these proteins are presently produced by *Escherichia coli* in the form of cytoplasmic aggregates or inclusion bodies, which contain protein that is misfolded and therefore functionally inactive (3). To improve the recovery of biologically active protein, the polypeptide in the inclusion bodies is first partially or completely unfolded in denaturants such as urea or guanidine hydrochloride. The denatured protein is then refolded by removal of the denaturant which is commonly performed by dialysis or dilution. During refolding, many proteins tend to aggregate, which causes significant reduction in the yield of active protein (3). A number of *in-vitro* aggregation inhibitors or folding aids such as polyethylene glycol (4), polyamino acids (5), sugars (6) and surfactants (7) have been reported to prevent aggregation and enhance protein refolding. An optimal folding aid would have several

* To whom correspondence should be addresed at Dow 346, Department of Chemistry, Central Michigan University, Mt. Pleasant, Michigan 48859. FAX:(517)774-2907.

Abbreviations: GuHCl, guanidine hydrochloride; CD, cyclodextrin; CAB, carbonic anhydrase B.

0006-291X/95 $5.00

critical properties (2). It must be cost effective. It should inhibit protein aggregation without adversely affecting the formation of native protein. Finally, it must be easily separated from the refolded protein. In the following investigation, we report the use of cyclodextrins as folding aids for a model protein, carbonic anhydrase.

MATERIALS AND METHODS

Reagents: Carbonic anhydrase II (from bovine erythrocytes, also designated as carbonic anhydrase B) and p-nitrophenol acetate were obtained from Sigma Chemical Co.(St. Louis,MO). Alpha-cyclodextrin, hydroxypropyl beta-cyclodextrin and gamma-cyclodextrin were obtained from American Maize-Products Company (Hammond, IN.,USA). The hydroxypropyl derivative of β-CD was used instead of β-CD due to the poor solubility of the latter in aqueous solutions. Guanidine hydrochloride was obtained from Life Technologies, Inc.(Gaithersburg, MD).

Protein concentration: Protein concentration of native CAB in 50 mM Tris-sulfate at pH 8.5 was determined by its absorbance at 280 nm and using an extinction coefficient of 1.83 $(mg/ml\ protein)^{-1}\ cm^{-1}$ and a molecular weight of 30,000 (8).

Esterase activity of CAB: The enzymatic avtivity of CAB was measured by its hydrolysis of the substrate, p-nitrophenol acetate (50mM Tris-sulfate, pH 7.5, concentration of substrate in assay mixture was 1 mM), forming p-nitrophenol, which was monitored at 400 nm and at 25°C in a double-beam spectrophotometer (Beckman Model 34). Assays were performed at pH 7.5 since the blank rates increase appreciably at higher pH. At the concentrations used in this investigation, there was no significant hydrolysis of the substrate by any of the three CDs.

Turbidimetric measurements: Turbidimetric analysis of protein aggregation was performed at 400 nm and 25°C on the Beckman Model 34 spectrophotometer.

Fluorescence measurements: Fluorescence spectra were obtained with a Perkin Elmer Luminescence Spectrometer LS50B.

CAB denaturation/renaturation: Carbonic anhydrase was denatured by overnight incubation in 5-7 M guanidine hydrochloride in 20 mM Tris-sulfate, pH 8.5 at 25°C. Protein inactivation was confirmed by enzymatic activity as well as fluorescence measurements. Refolding of carbonic anhydrase was conducted by rapid dilution in renaturation buffer consisting of 50 mM Tris-sulfate, pH 8.5.

RESULTS

When denatured CAB in GuHCl,was rapidly diluted with 50mM Tris-sulfate buffer at pH 8.5, to 1.7 μM protein and 0.03 M GuHCl, aggregation was observed immediately and was monitored by light scattering at 400 nm (Figure 1, curve A). Aggregation increased with time and ther stabilized after approximately 5 minutes. When the denatured protein was renatured in the presence of CDs under the same conditions, light scattering due to aggregation was significantly reduced. The ability of cyclodextrins to inhibit CAB aggregation was in the order of α-CD > hydroxypropyl β-CD > γ-CD (Figure 1, curves D, C and B, respectively). Inhibition of CAB aggregation was enhanced with increasing concentration of cyclodextrin in the renaturation buffer (data not shown).

Typical results of the reactivation kinetics of CAB at aggregating conditions (17 μM CAB, 0.34 M GuHCl) in the presence of alpha, beta and gamma cyclodextrins is shown in Fig. 2. Denatured CAB in GuHCl was diluted with renaturation buffer containing 100

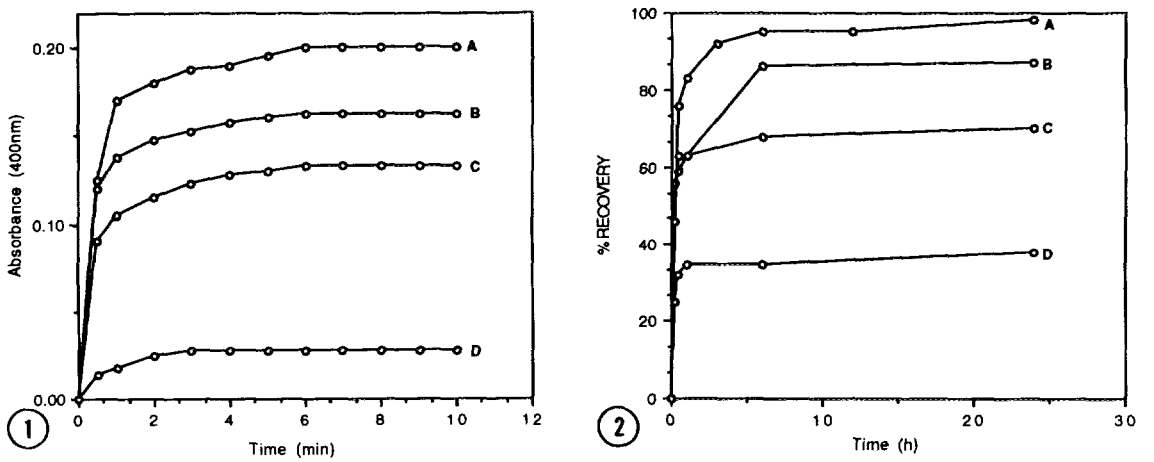

Fig. 1.
Aggregation kinetics in the absence and presence of cyclodextrins. Native enzyme (33mM) was denatured overnight in 6.8M GuHCl. In each case, protein was diluted to 1.7 μM and 0.034M GuHCl, with renaturation buffer, containing 50mM CD. Curve A (absence of CD), Curve B (γ-CD), Curve C (β-CD) and Curve D (α-CD).

Fig.2.
Reactivation kinetics in the presence of cyclodextrins. Denatured CAB (333 μM) was diluted to 17 μM protein and 0.34M GuHCl with 50mM Tris-sulfate buffer, pH 8.5, containing 100mM cyclodextrin. Curves A, B, C, show the renaturation kinetics obtained with α-CD, β-CD and γ-CD, respectively. Control without cyclodextrin is shown as Curve D.

mM CD at 25°C. Recovery of esterase activity (compared to the native enzyme) was then measured as a function of time after dilution. The rate of renaturation was rapid in the first few minutes and then gradually reached a plateau within 6 hours of renaturation. Highest recovery of activity was achieved with α-CD, followed by hydroxypropyl β-CD and γ-CD (Fig.2, curves A, B and C, respectively). In the absence of cyclodextrin, the recovery of enzyme activity is only about 40 % (Fig.2, curve D). Alpha-cyclodextrin at 100 mM gave over 80 % recovery in less than an hour.

CAB renaturation kinetics in the presence of varying amounts of α-CD (0-100 mM) in the dilution buffer was assessed at 17 μM protein and 0.34 M GuHCl (Fig.3). Although the initial rates of recovered activity did not increase appreciably, the final yields of reactivated enzyme obtained, however, increased with increasing amounts of alpha-CD.

Effect of protein concentration on reactivation efficiency at 0.1M alpha-CD is shown in Fig.4. In each case, refolding was allowed to occur for 6 hours. Over 90 % recovery was obtained for CAB concentration as high as 67 μM. Recovery of active protein decreased at higher protein concentrations due to increased protein aggregation.

The effect of pH and temperature on cyclodextrin assisted CAB refolding is summarized in Table 1. In each case,the enzyme was refolded by dilution to 17 μM protein and 0.34M GuHCl for 6 hours. Optimal yield of active protein was obtained between 25°C-37°C and under alkaline conditions.

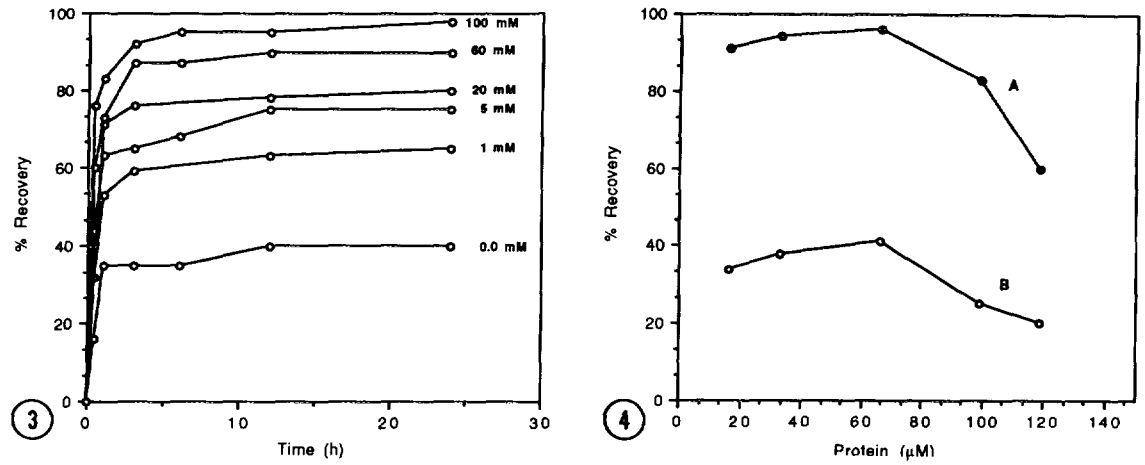

Fig. 3.

Refolding kinetics in the presence of varying amounts (0-100 mM) of α-CD. Conditions for denaturation / renaturation are similar to those described in Fig.2. In each case, CAB concentration during renaturation was 17 μM.

Fig.4.

Effect of protein concentration on refolding kinetics of CAB. Native protein (1.2 mM) was denatured in 6.8 M GuHCl overnight. The denatured enzyme was diluted to 17μM-122 μM protein and 0.68 M GuHCl. Curve A represents enzyme recovery obtained with 100 mM α-CD while Curve B repesents recovery obtained in its absence.

DISCUSSION

Cyclodextrins are non-toxic, doughnut shaped, cyclic macromolecules which typically consists of six to eight α1-4 linked glucose units. In the centre of the molecule is a 6-10 A^0 hydrophobic cavity that is capable of forming "inclusion complexes" with hydrophobic guest molecules of appropriate dimensions. With respect to proteins, aromatic amino acids such as tryptophan and phenylalanine, are known to form weak inclusion complexes with CDs (9).

Earlier work on CAB refolding (10) has shown that it aggregates at low denaturant concentrations (0.2 to 0.8 M GuHCl) and high protein concentrations (>3 μM). Refolding of CAB proceeds through an intermediate species, the first observed intermediate which contains exposed hydrophobic clusters. This intermediate may form dimers and micron size aggregates under appropriate conditions (10).

Our laboratory has been actively involved in investigating the interactions of cyclodextrins with various biomolecules such as cholesterol (11), lipoproteins (12) and proteins (13). The concept of utilizing cyclodextrins as protein folding agents was inspired by these studies.

As is evident from the results above, refolding of CAB in the presence of cyclodextrins leads to enhanced recovery of active enzyme. Turbidimetric studies demonstrate that alpha-CD is a better inhibitor of CAB aggregation than the beta or gamma oligosaccharide. This

TABLE I
Effect of pH and temperature on CD enhanced CAB refolding

		% Recovery	
pH	Temp(°C)	- αCD	+ αCD
8.5	4	19	75
8.5	25	30	90
8.5	37	32	95
8.5	50	0	1
5.0	25	0	0
6.0	25	14	65
7.0	25	30	87
8.0	25	28	88
9.0	25	24	84

may be due to better fit of aromatic amino acids in α-CD's cavity. The importance of this interaction between CD and the hydrophobic sites in CAB intermediate(s), is demonstrated by competitive studies shown in Fig. 5. As is the case in Fig. 1, dilution of denatured CAB resulted in aggregate formation which increased light scattering at 400 nm (curve A). When dilution was performed in α-CD, aggregation was inhibited (curve D). However when dilution was performed in the presence of CD and an aromatic amino acid (Trp, curve B, Phe, curve C), protein aggregation increased. On the other hand, a polar amino acid such as glycine showed no effect on the aggregation kinetics (data not shown). These results suggest that α-CD forms inclusion complexes with exposed hydrophobic sites such as aromatic amino acids present in the protein folding intermediate(s). This "blocking" of the "sticky" sites during CAB refolding by CDs is a possible mechanism of CD-induced inhibition of protein aggregation.

If the CD-folding intermediate interaction was too strong, the CDs will be trapped in the protein as it folds on denaturant removal. That this is not the case is evident from the high recovery of enzyme activity obtained in the presence of cyclodextrin. CD renatured protein also shows identical fluorescence spectra as that of the native enzyme (Fig. 6). These results indicate that the relatively polar cyclodextrin molecules that are weakly bound to hydrophobic sites in the folding intermediate(s), are gradually removed as the interior of the protein becomes increasingly non-polar during protein refolding. In addition, the low molecular weight of cyclodextrins allows them ready access to and from the protein's interior during refolding.

We have demonstrated a novel strategy for protein reactivation by using a water-soluble, low molecular weight binding agent that forms weak, non-covalent complexes with hydrophobic sites present in protein folding intermediate(s). The above characteristics of cyclodextrins allows them to inhibit aggregation without interfering with

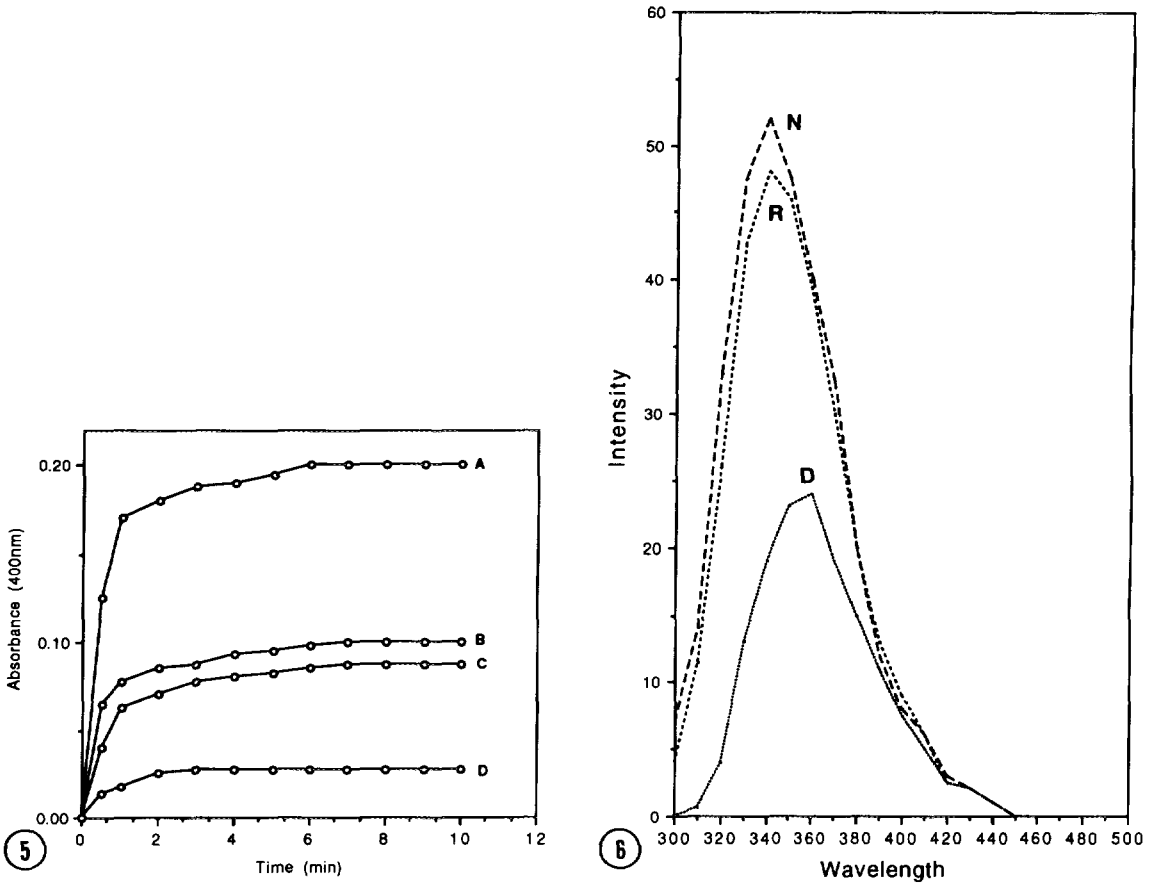

Fig. 5.
Aggregation kinetics of CAB in the presence of cyclodextrins and competitors. Denatured CAB was diluted to 1.7 μM protein and 0.034M GuHCl. Curves A and D represent aggregation in the absence and presence of 50 mM α-CD, respectively. Curves B and C show aggregation observed in the presence of 50 mM α-CD plus competitor (Trp, 2.5 mM and Phe, 3 mM, respectively.

Fig. 6.
Fluorescence spectra of CAB. Curve N represents the spectrum of the native protein in 50 mM Tris-sulfate, pH 8.5. Curve D represents the spectum of denatured CAB in 6.8 M GuHCl while Curve R is the spectrum of the protein renatured with 100 mM α-CD for 1 h . In each case the final protein concentration was 0.56 μM. All spectra have been corrected for their respective blanks.

protein refolding, thus making them ideal protein folding agents. In addition, CDs are relatively inexpensive, commercially available and are easily separated, if necessary, from the refolded protein by dialysis or gel filtration.

ACKNOWLEDGMENT

This work was supported by a REF Grant #627-31 from the State of Michigan.

REFERENCES

1. Anfinsen, C. B., Haber, E., Sela, M. and White, F. H. (1961). Proc. Nat. Acad. Sci., 47, 1309-1314.
2. Cleland, J. L. (1993) in Protein Folding in Vivo and In Vitro (Cleland,J.L., Ed.) pp 1-21, American Chemical Society, Washington, D.C.
3. Marston, F. A. O. (1986) Biochem J. 240, 1-12.
4. Cleland, J. L. and Wang, D.I.C. (1990) Biotechnology 8, 1274-1278.
5. Schaffer, S. W., Ahmed, A. and Wetlaufer, D. B. (1975) J. Biol. Chem. 250, 8483-8486.
6. Valax, P. and Georgiou, G. (1991) in Protein Refolding (Georgiou,G., DeBernardez-Clark, E., Eds.) pp 97-109, American Chemical Society, Washington, D.C.
7. Tandon, S. and Horwitz, P. M. (1987) J. Biol. Chem. 262, 4486-4491.
8. Wong, K. P. and Tanford. C. (1973) J. Biol. Chem. 248, 8518-8523.
9. Wood, D. J., Hruska, F. and Saenger, W. (1977) J. Am. Chem. Soc. 99, 1735-1740.
10. Cleland, J, L. and Wang, D. I. C. (1990) Biochemistry 29, 11072-11078.
11. Karuppiah, N., Kaufman, P. B., Kapustka, S. and Sharma, A. (1993) Microchem. J. 47, 325-329.
12. Sharma, A. and Janis L. (1991) Clin. Chim. Acta 199, 129-138.
13. Xu, Pei-pei, Troupe, C. M. and Sharma, A. (1994) Microchem. J. 49, 85-90.