Effects of monopropanediamino-β-cyclodextrin on the denaturation process of the hybrid protein BlaPChBD

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A B S T R A C T
Irreversible accumulation of protein aggregates represents an important problem both in vivo and in vitro. The aggregation of proteins is of critical importance in a wide variety of biomedical situations, ranging from diseases (such as Alzheimer’s and Parkinson’s diseases) to the production (e.g. inclusion bodies), stability, storage and delivery of protein drugs. β-Cyclodextrin ([β-CD]) is a circular heptasacharide characterized by a hydrophilic exterior and a hydrophobic interior ring structure. In this research, we studied the effects of a chemically modified β-CD (BDC07056), on the aggregating and refolding properties of BlaPChBD, a hybrid protein obtained by inserting the chitin binding domain of the human macrophage chitotriosidase into the class A β-lactamase BlaP from Bacillus licheniformis 749/1 during its thermal denaturation. The results show that BDC07056 strongly increases the refolding yield of BlaPChBD after thermal denaturation and constitutes an excellent additive to stabilize the protein over time at room temperature. Our data suggest that BDC07056 acts early in the denaturation process by preventing the formation of an intermediate which leads to an aggregated state. Finally, the role of β-CD derivatives on the stability of proteins is discussed.

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1. Introduction

Protein aggregation is an important phenomenon in biotechnological processes as well as in human diseases [1,2]. In many cases, aggregation occurs when proteins, under particular conditions, fail to fold correctly or to remain correctly folded. This is a common issue encountered both in vivo and in vitro. In vivo, protein aggregation can result in a wide range of diseases, including amyloidoses (i.e. Alzheimer’s and Parkinson’s diseases, and spongeform encephalopathy), which are associated with the deposition of proteins into highly structured aggregates into a variety of tissues.

Protein aggregation results from many causes such as prolonged thermal treatments and incorrect folding during protein expression, purification, formulation, transportation, or storage. A growing number of peptides and protein are being used in therapy, the application of many of them is however hindered by unfavorable solubility, instability or aggregation [3]. Hence, the ability to prevent or reverse protein aggregation is vital to the production and formulation of therapeutic proteins.

Many attempts have been undertaken to overcome in vitro protein aggregation by using a variety of cosolutes such as L-arginine [4–7], polyethylene glycol (PEG) [8,9] and cyclodextrins [3,10]. Cyclodextrins are circular oligosaccharides composed of α−(1,4) linked α−β−γ−cyclodextrins consist of six, seven and eight glucosyl units. The salient characteristic of a cyclodextrin molecule is the presence of a central “cavity” or “hole” which provides an excellent resting site for hydrophobic molecules of appropriate dimensions [1,11]. The α−, β− and γ-cyclodextrins consist of six, seven and eight glucosyl units, respectively, β-cyclodextrins ([β-CD]), which represent the most abundant class, have a rather limited solubility in water (about 18 g/L) [12] and various derivatives have been synthesized to improve it [1,13].

Studies on cyclodextrins have essentially focused on their ability to prevent aggregation during the renaturation step of proteins expressed as inclusion bodies in their hosts. In a typical experiment, purified inclusion bodies are solubilized in highly concentrated solutions of chaotropic agents (e.g. urea, guanidinium chloride), and then the denatured proteins are refolded by diluting the denaturant [14]. The yield of refolding is often limited by protein aggregation [4,14,15], and can be increased by adding β-CD in the refolding buffer.

Abbreviations: ChBD, chitin binding domain of the human macrophage chitotriosidase; BDC07056, monopropanediamino-β-cyclodextrin; PEG, polyethylene glycol; Far UV-CD, circular dichroism in far ultraviolet; β-CD, β-cyclodextrin; Tm, temperature of mid-transition; ANS, 1-Anilino-8-naphthalenesulfonate

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as shown for bovine carbonic anhydrase [1], α-amylase [16,17], aminocyclase [18], human growth hormone [19] and insulin [20].

In a recent report, we have described the insertion of the chitin binding domain (ChBD) of the human macrophage chitotriosidase into a solvent-exposed loop of a class A β-lactamase (BlaP) produced by Bacillus licheniformis 749/[21,22]. The resulting hybrid protein, referred to as BlaPChBD, was constructed in order to study the structural and functional reciprocal effects of an insertion on the scaffold protein and a heterologous structured protein fragment. This hybrid protein behaves as a soluble protein and conserves both the chitin binding property and β-lactamase activity of the parental proteins. Compared to the parental β-lactamase, the thermal unfolding of the hybrid protein is not cooperative and involves the formation of stable intermediate species which has the tendency to aggregate [21].

In the present study, we have investigated the effects of a chemically modified β-CD, monopropanediarnino-β-cyclodextrin (called BCD07056), on the inactivation time course of BlaPChBD at room temperature and on its thermal unfolding, using a variety of techniques including fluorescence, UV-absorbance, far UV CD and mass spectrometry. The results of this study show that the addition of BCD07056 enhances the life time of the protein at room temperature, restores a cooperative thermal unfolding, allows the reversibility of the thermal denaturation and prevents protein aggregation.

2. Materials and methods

2.1. Cyclodextrin

BCD07056 was produced and kindly provided by Biocydex (France). This modified β-cyclodextrin is also known as 6-(3-aminopropylamino)-6-deoxyxylomaltoheptaose and contains one modification per glucose ring. Different cyclodextrin concentrations (1 mM, 10 mM and 100 mM) have been used in thermal denaturation experiments of BlaPChBD. 10 mM was shown to be the optimal concentration did not result in an increase of the protein stability. This hybrid protein behaves as a soluble protein and conserves both the chitin binding property and β-lactamase activity of the parental proteins. Compared to the parental β-lactamase, the thermal unfolding of the hybrid protein is not cooperative and involves the formation of stable intermediate species which has the tendency to aggregate [21].

2.2. Expression and purification of the chimeric protein BlaPChBD

The experimental procedures have been described [21].

2.3. Enzymatic activity assays

The β-lactamase activity of purified proteins was measured with nitrocefin (initial rate) and cephalothin (kinetic parameters determination) as substrates in 50 mM sodium phosphate, 150 mM NaCl, pH 7.5 at 25 °C. Specific activities, $k_{cat}$ and $K_m$ values of the purified enzymes were determined as described by Matagne et al. [23].

2.4. Fluorescence studies

Fluorescence spectra of proteins were recorded with a SML-A MINCO Model 8100 spectrofluorimeter (Spectronic Instruments) in a 1 cm optical pathlength quartz cuvette in 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.5 with or without 10 mM BCD07056 with a protein concentration of 2.6 μM. The excitation wavelength was 280 nm. The fluorescence spectra were recorded from 300 to 400 nm and corrected for the fluorescence of buffered solutions.

For fluorescence spectra of aromatic amino acids solutions, the excitation wavelengths were 280 nm, 274 nm and 257 nm to specifically target Trp, Tyr and Phe, respectively. Their fluorescence spectra were recorded from 300 to 400 nm for Trp, from 284 to 384 nm for Tyr and from 274 to 374 for Phe. The amino acid concentration was 100 μM.

Heat-induced transitions were monitored by measuring the intrinsic fluorescence of the protein solution. The temperature was increased from 25 °C to 90 °C at a rate of 0.5 °C/min using a programmed Lauda Ecoline RE306 water bath. Temperature in the cell was checked using a thermometer. The used excitation and emission wavelengths were 280 and 337 nm respectively. Data were normalized using the pre- and post-transition baseline slopes as described [24,25]. The buffer composition as well as protein concentrations were exactly the same as described for fluorescence spectra.

When heat-induced unfolding was monitored in the presence of ANS, emission was recorded at 475 nm with excitation at 350 nm. Protein (2.4 mM) was incubated in the presence of 345 μM ANS ($\lambda_{ex}(350 \text{ nm})\ \lambda_{em}(4950 \text{ M}^{-1} \text{ cm}^{-1})$). Data were corrected for the background fluorescence of the solution in the absence of protein (buffer + ANS). The temperature was increased from 25 °C to 90 °C at 1 °C/min, and data were collected as described above.

2.5. Circular dichroism

Heat-induced unfolding transitions were monitored by far-UV CD were recorded with a Jasco J-810 spectropolarimeter, equipped with a Peltier holder at 222 nm. Temperature in the 0.1-cm pathlength cell was checked using a thermocouple and corrected. Experiment conditions were the same as indicated above and the temperature was increased from 25 °C to 97 °C at a rate of 0.5 °C/min using a protein concentration of about 2.6 μM in 50 mM phosphate buffer, 150 mM NaCl, pH 7.5. Data were acquired every 0.2 °C, with a four second integration time and a 2 nm bandwidth. Data were normalized using the pre- and post-transition baseline slopes as described [25].

2.6. $K_r$ determination

Binding assays were conducted as follows: various concentrations of the chimeric protein (25 nM to 3 μM) were incubated in the presence of 10 mg of chitin in final volume of 500 μl of 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.5 at 22 °C with continual mixing. The mixtures were centrifuged at 4 °C for 15 min at 13,000 rpm and the supernatant containing the free protein was collected. The free protein concentration was determined using the reporter β-lactamase activity. The amount of bound protein was calculated as the difference between the initial protein concentration and the free protein concentration after binding. The relative equilibrium association constant ($K_r$) was determined by the method described by Gilkes et al. [26] using the following equation:

$$[B] = \frac{[N_0]K_r[F]}{1 + aK_r[F]}$$

Where $[B]$ is the concentration of bound ligand (moles. g chitin$^{-1}$), $[F]$ the concentration of free ligand (molar), $[N_0]$ the concentration of binding site in the absence of ligand, a the number of lattice units occupied by a single ligand molecule and $K_r$ the equilibrium association constant (L mol$^{-1}$). The $K_r$ value cannot be isolated from this equation but the relative equilibrium association constant, $K_r$ (L g chitin$^{-1}$) is defined as:

$$K_r = \frac{[N_0]}{K_s}$$

2.7. Light scattering

The scattering analysis of protein aggregation was performed at 25 °C on an Uvikon XS spectrophotometer (Bio-Tek Instruments). The apparent absorbance obtained was measured at 490 nm in a quartz cell of 1-cm path length.
2.8. Solubility studies of aromatic amino acids

Excess quantities of Phe, Trp or Tyr (Sigma) were added to 1 mL Eppendorf tubes containing various concentrations of BCD07056 (BioCydex, France) at a neutral pH. The tubes were shaken until equilibrium was reached (24 h). Thereafter, the solutions were filtered through 0.45 μm PVDF filter and the concentration of either Phe, Trp or Tyr was spectrophotometrically determined using a Beckman Coulter™ (DU® 530) spectrophotometer at either 257, 280 or 274 nm respectively. For each spectrophotometric determination a solution corresponding to the same concentration of BCD07056 was used as a blank. The experiments were carried out in duplicate at least three times independently and an average value is presented.

2.9. Mass spectrometry

ESI-TOF mass spectrometry experiments were realized in 20 mM acetonitril pH 7.4. The protein and β-cyclodextrin concentrations were 5 μM and 10 μM, respectively.

3. Results

3.1. Conservation of the biological activities of BlaPChBD in the presence of BCD07056

The effects of BCD07056 on both the β-lactamase activity and the chitin binding property of BlaPChBD were investigated. The kinetic parameters of the chimeric protein were measured in the presence or in the absence of BCD07056 (10 mM) using cephalolin as substrate (Table 1). The data suggest that the presence of BCD07056 slightly increases the enzymatic activity, the $k_{cat}/K_M$ value is increased by three fold, mostly due to a two fold decreased of the $K_M$ value. To analyze the effect of BCD07056 on the functionality of the inserted ChBD, we determined the relative equilibrium association constant ($K_r$) between BlaPChBD and chitin using the method described by Gilkes et al. [26]. The calculated $K_r$ values in the absence and in the presence of BCD07056 are 5.4 ± 0.5 L g⁻¹ and 5.6 ± 0.6 L g⁻¹, respectively. These results indicate that no alteration of the chitin binding property of the chimeric protein is observed in the presence of BCD07056.

3.2. Ability of BCD07056 to stabilize BlaPChBD during its storage at room temperature

To investigate a possible stabilization effect of BCD07056 during the storage of BlaPChBD, samples of the protein (320 nM) were incubated in the presence and in the absence of 10 mM BCD07056, at 24 °C for three weeks. Each week, the initial rate of nitrocefin hydrolysis was measured and normalized to the value obtained at time 0 (Fig. 1). After three weeks, the hybrid β-lactamase conserved 73% of its initial enzymatic activity when incubated in the presence of BCD07056, whereas when the enzyme was incubated for the same period of time without the molecule, substrate hydrolysis was barely detectable. These results show that BCD07056 is able to limit significantly the inactivation of the protein during is storage at room temperature.

<table>
<thead>
<tr>
<th>BCD07056</th>
<th>$K_M$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_M$ (μM⁻¹ s⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>– BCD07056</td>
<td>77 ± 1</td>
<td>75 ± 5</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>+ BCD07056</td>
<td>35 ± 2</td>
<td>98 ± 5</td>
<td>2.8 ± 0.5</td>
</tr>
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Addition of BCD07056 to the partial inactivated enzyme (i.e. enzyme incubated for 2 weeks at 24 °C) did not lead to any increase of the enzymatic activity (data not shown) suggesting that BCD07056 can limit enzyme inactivation but cannot reverse it.

3.3. Effects of BCD07056 on the thermal stability of BlaPChBD

Previously, we have reported that the thermal denaturation of BlaPChBD is a non-cooperative process involving the formation of an intermediate species between the native (N) and the unfolded (U) states. Moreover, the thermal unfolding of BlaPChBD is only partially reversible and involves aggregation [21].

The effects of BCD07056 on the thermal denaturation behaviour of the protein were investigated by fluorescence and far UV CD spectroscopies (Fig. 2). The transition curves monitored by intrinsic fluorescence exhibit an apparent single transition both in the absence and in the presence of BCD07056, with apparent mid-transition ($T_M$) temperatures of 53.1 ± 0.1 °C and 56.9 ± 0.5 °C, respectively (Fig. 2(b)). The weak increase of the $T_M$ value in the presence of BCD07056 indicates that this molecule has a moderate effect on the thermal stability of BlaPChBD.

The data obtained by far UV CD show that the denaturation of BlaPChBD in the presence of BCD07056 also follows an apparent single transition ($T_M$ 57.1 ± 0.8 °C). The normalized data obtained by fluorescence (tertiary structures) and far UV CD (secondary structures) in the presence of the modified β-CD are virtually superimposable, suggesting that thermal unfolding of the chimeric protein follows a two state transition without populating any detectable intermediate species between N and U. This observation differs from that in the absence of BCD07056; in this case, the curve obtained exhibits two transitions (Fig. 2(a)) with apparent $T_M$ values of 53.3 ± 0.7 °C and 67.6 ± 0.3 °C.

To investigate the reversibility of the thermal unfolding of BlaPChBD in the presence or in the absence of BCD07056, fluorescence spectra before and after a complete cycle of thermal denaturation/renaturation were monitored (Fig. 3). In the absence of BCD07056, only about 50% of the spectroscopic signal is recovered after a complete denaturation/renaturation cycle. In the presence of BCD07056, the fluorescence of the refolded state is very similar to that of the native state indicating that thermal denaturation is reversible in the presence of the molecule. These results are further supported by the enzymatic activity measurements. The yields of recovered β-lactamase activity in the absence or in

![Fig. 1. Evolution of the enzymatic activity of BlaPChBD (320 nM) incubated at 24 °C in function of time, in the presence (●) or in the absence (○) of 10 mM BCD07056. Nitrocefin (100 μM) hydrolysis was measured in 50 mM phosphate buffer, 150 mM NaCl, pH 7.5 in the presence of 0.64 mM BlaPChBD.](image-url)
the presence of BCD07056 were 51±15% and 91±7%, respectively (data not shown).

\[ K_r \] determinations were also performed after renaturation of the protein. Without BCD07056, only 48% of the \[ K_r \] value was restored. In contrast, the presence of BCD07056 allows the complete recovery of this value.

Next, we have studied the aggregation behaviour of the chimeric protein after one cycle of thermal denaturation/renaturation by light scattering at 490 nm. A strong increase in scattering is measured with a BlaPChBD sample heated to 80 °C and cooled at room temperature in the absence of BCD07056 (Fig. 4(a)). In contrast, no significant increase in turbidity is detected when the protein denaturation/renaturation cycle is performed in the presence of 10 mM BCD07056, suggesting that this molecule prevents BlaPChBD aggregation.

To analyze in more detail the aggregation phenomenon during the thermal unfolding of BlaPChBD in the absence and in the presence of BCD07056, the protein was heated at a rate of 0.5 °C/min and the absorbance at 490 nm of aliquots taken every 5 °C were measured. The data presented in Fig. 4(b) suggest that aggregation observed in the absence of BCD07056 occurs late in the transition (at ±75 °C), namely 18 °C above the formation of the intermediate state observed by far UV CD and ANS fluorescence (Fig. 2). This observation suggests that the aggregation does not involve directly the intermediate state [21] but rather the unfolded state. But it has to be noticed that herein we did not take into account any kinetic effects and that the intermediate populated at lower temperature could exhibit low aggregation kinetic and be responsible of the observed aggregation.

Furthermore, when the addition of BCD07056 is performed at different temperatures during the thermal denaturation transition (Fig. 2), namely at 52 °C (when the intermediate state is still populated in minority), at 58 °C (when the intermediate state is highly populated) or at 65 °C (when the denatured state begins to be mostly populated), the molecule is not as efficient as when it is added at 25 °C (Table 2). This indicates that BCD07056 does not interact with the denatured state and probably acts early in the denaturation process.

3.4. Interaction between BlaPChBD and BCD07056

It has been proposed that the ability of cyclodextrins to bind to the side chains of aromatic amino acids contributes to their anti-aggregating effects on proteins [3,18,19]. The fluorescence properties of aromatic residues are sensitive to the microenvironment [27]. Thus
the binding of BCD07056 to aromatic residues was studied using free Tryptophan (Trp), Phenylalanine (Phe) and Tyrosine (Tyr) as models. The intrinsic fluorescence spectra of the three aromatic amino acids in the presence of increasing concentrations of BCD07056 are presented in Fig. 5. The data show that the intrinsic fluorescence of free Trp increases after the addition of BCD07056. A blue shift (350 nm to 343 nm) of the maximum emission wavelength of Trp, characteristic of decreased solvent access is observed. But this blue shift might also be partly due to the fact that the Trp emission spectra become broader in the presence of BCD07056. Kim and co-workers observed similar results with the natural β-CD [18]. The intensity of the intrinsic fluorescence emission of Phe also increases with BCD07056 concentrations. In contrast, the fluorescence of Tyr decreases with increasing BCD07056 concentrations suggesting a fluorescence quenching of this residue by the β-cyclodextrin. These results indicate that the addition of BCD07056 to solutions of free aromatic amino acids leads to a micro-environmental change of the aromatic side-chains due to a probable docking of the latter into the hydrophobic cavity of BCD07056 molecules. These data are supported by the solubility assays performed on the three aromatic residues (Fig. 6) which give a crude measure of the interaction between the free aromatic residues and the cyclodextrin. The curves were obtained by plotting the absorbance of the filtered amino acid solutions at specific wavelengths in function of BCD07056 concentrations. From these experiments, we can conclude that BCD07056 increases the solubility of the three aromatic amino acids.

In order to detect any interaction between BCD07056 and the native BlaPChBD, intrinsic fluorescence spectra of the protein were recorded in the presence of the molecule upon excitation at 295 nm, 274 nm and 257 nm to specifically target Trp, Tyr and Phe residues, respectively. The results (not shown) demonstrated that addition of 10 mM BCD07056 did not affect the intrinsic fluorescence of the protein solution whatever the excitation wavelength was. We also compared the intrinsic fluorescence spectra of the thermal denatured form of the protein in the presence or in the absence of BlaPChBD, no change was observed (not shown). This indicates that the microenvironments of the aromatic side-chains of the protein are not significantly changed suggesting that BCD07056 does not interact with either the native state or the denatured state of the hybrid protein. This observation is in good agreement with prediction of the solvent accessibility of aromatic amino acids into BlaPChBD. The parental β-lactamase contains 3 Trp, 6 Tyr, and 6 Phe whereas ChBD contains 1 Trp, 2 Tyr, and 4 Phe. By using the crystallographic 3D structure of BlaP (4BLM, RCSB PDB) [28], we were able to make a prediction of the solvent accessibility of its aromatic residues with the Swiss-Pdb viewer 3.7 program [29]. With the exception of three poorly solvent exposed Tyr, all the other aromatic amino acids are buried in the hydrophobic core of the protein. The 3D structure of ChBD has not been solved yet.

The interaction between BCD07056 and the hybrid protein was further studied by monitoring the fluorescence of 1-Anilino-8-naphthalenesulfonate (ANS). ANS is used widely as a conformational probe of hydrophobic patches in proteins [30]. Results of ANS binding experiments realized in the presence or in the absence of BCD07056 are compared in Fig. 7. As described in our previous work [21], in the absence of the β-CD, the thermal denaturation of BlaPChBD was accompanied by a substantial increase of ANS binding up to about 57 °C indicating that thermal alterations of the BlaPChBD tertiary structure occurred with the formation of ANS-accessible hydrophobic clusters at the surface of the intermediate species. On the contrary, no binding of ANS is observed when the thermal denaturation is performed in the presence of β-CD. These data indicate that the accessibility of hydrophobic clusters into BlaPChBD is dramatically decreased in the presence of BCD07056. In contrast, the fluorescence of Tyr increases with increasing BCD07056 concentrations suggesting a fluorescence quenching of this residue by the β-cyclodextrin. These results indicate that the addition of BCD07056 has been added at different temperatures during the thermal denaturation (10 mM final concentration). The recovered activity was determined by measuring the rate of nitrocefin hydrolysis expressed as %mole of nitrocefin hydrolyzed/min/mg of protein. The intrinsic fluorescence of Tyr decreases with increasing BCD07056 concentrations suggesting a probable docking of the latter into the hydrophobic cavity of BCD07056 molecules.
presence of the $\beta$-CD suggesting either an interaction between the protein and the molecule or a more cooperative behaviour of the protein during thermal denaturation.

4. Discussion

Protein aggregation is considered as acting in competition with the protein folding pathway. This concept was derived from the fact that protein aggregation is a common property of polypeptide chains and that the process often takes place from at least partially unfolded state [31,32]. Generally, the native protein and aggregates can be seen as originating from a common population of partially unfolded, interconverting molecules which interact with either the solvent or neighboring molecules. In other words, protein aggregation and protein folding are two sides of the same coin, and it could be argued that structural factors playing relevant roles in one process are also involved in the other [31].

The efficiency of protein folding is determined by the respective rates of folding and aggregation [18,33,34]. To prevent protein aggregation, various low-molecular-weight chemical additives have been successfully used [35–37]. Among them, cyclodextrins have shown to be efficient for the correct refolding of a number of proteins [1,3,16,19].
efficiency of cyclodextrins is proven to be dependent on the cavity size and the nature of the chemical modifications found on the ring of the sugar molecules [1].

In this work, we investigated the effect of a chemically modified β-CD (BCD07056) on the stability and aggregation behaviour of the hybrid protein BlaPChBD. Until now, most studies performed in the presence of cyclodextrins have focused on their anti-aggregating effect during the refolding process of chemically denatured proteins. Here, we specifically monitored the effects of a chemically modified β-cyclodextrin on the thermal unfolding process of BlaPChBD and enzyme inactivation during long storage at room temperature.

The results obtained by incubating BCD07056 with the chimeric protein at 24 °C for three weeks indicated that the presence of BCD07056 significantly limits the inactivation of the protein. But if BCD07056 is added after the inactivation of the chimeric protein, no significant recovery of enzyme activity is observed. This suggests that BCD07056 can prevent the inactivation of BlaPChBD but cannot reverse it. Thus, BCD07056 constitutes a suitable additive to maintain protein stability during storage.

Many studies have reported interactions between cyclodextrins and proteins via exposed aromatic residues [1,3,19]. It is reasonable to assume that interactions between cyclodextrins and proteins are likely to occur during protein denaturation, since it is during the unfolding process that most of the buried hydrophobic residues of the polypeptide become solvent-exposed [1]. Our results support this hypothesis since our experiments do not allow the detection of direct interactions between BCD07056 and the native form of BlaPChBD.

In a previous work, we reported that thermal denaturation of BlaPChBD was accompanied by the formation of an intermediate state (I) which exhibits ANS-accessible hydrophobic clusters. In the present study, we show that aggregation begins to occur at 70 °C. At this temperature, the mostly populated state is the unfolded state (U). This suggests that U could be involved in the aggregation phenomenon instead of (I) as we previously proposed [21] (although long aggregation kinetics could also occur involving species populated earlier than U in the denaturation process). Thus, we propose that, in the absence of BCD07056, the thermal denaturation pathway favors the formation of the intermediate state (I) and then the unfolded state (U) which tends to precipitate irreversibly and to form the aggregated state (A) (Scheme 1). In the presence of BCD07056, the superposition of the denaturation transitions monitored by intrinsic fluorescence and far UV CD indicates that the denaturation of secondary and tertiary structures occurs simultaneously. This demonstrates that thermal denaturation of BlaPChBD in the presence of the molecule occurs without populating any stable intermediates. Moreover, in addition to restore the cooperativity of the thermal denaturation of the hybrid protein, the modified β-cyclodextrin also increases drastically its reversibility by preventing protein aggregation. Indeed, in the presence of the molecule, the yield of recovered enzymatic activity is 91 ± 7%.

In the case of cyclodextrins, stabilization of proteins involves necessarily an interaction between the macromolecule and the stabilizing agent. In our study, the data suggest that no stabilizing interaction occurs between BCD07056 and either the native state (N), the intermediate state (I) or the denatured state (U). Indeed, fluorescence spectra experiments suggest that no interaction occurs between the molecule and either the native state (N). An interaction between BCD07056 and the intermediate state (I) was also excluded because this one is not formed in the presence of the β-CD. And finally, since the addition of BCD07056 at 75 °C does not improve the reversibility of the thermal denaturation, and that no change in the fluorescence of the denatured state recorded with or without the cyclodextrin was observed, we excluded any interactions with the unfolded state (U/A).

Thus, we suggest that another intermediate species (I′) exists and is the target of BCD07056. This species probably presents solvent exposed aromatic amino acids which interact with the modified β-CD as soon as it is formed. We assume that this species appears early in the denaturation process. ANS binding experiments support an interaction between cyclodextrin and BlaPChBD during this denaturation process. However, it has to be noticed that the loss of ANS binding in the presence of the molecule may also be explained by the more cooperative behaviour of the protein in its presence. In the later case no ANS interaction would be observed because the denaturation of the protein would occur cooperatively without exposing hydrophobic patches able to bind ANS.

5. Conclusions

In conclusion, we hypothesize that in the absence of the molecule, the denaturation process of BlaPChBD is multi-state as shown in Scheme 1. When BCD07056 is present, the denaturation process is modified. When temperature begins to increase, the molecule interacts with aromatic residues of the species I′ as soon as they are solvent-exposed. This prevents the formation of the species I and leads to a modified denatured state (U′) which does not aggregate. Thus, BCD07056 could prevent exposure of hydrophobic clusters by producing a sort of “protective entrapment” which modifies the denaturation process and prevents aggregation of the protein. In summary, we proposed that in the absence of BCD07056 the irreversible pathway leading to protein aggregation is favored and that, in the presence of the molecule, the reversible pathway is mostly populated by the protein molecules.

An alternative hypothesis that could explain how BCD07056 stabilizes the hybrid protein would be that one or more non-detectable soluble intermediate(s) are formed in the early stage of the thermal denaturation process, these intermediates would exhibit low aggregation kinetics and would be the target of the modified cyclodextrin. The binding of BCD07056 to these intermediates would stabilize them and therefore prevent aggregation. In this alternative model the denaturation process would follow a single pathway that would include these aggregation-prone intermediates in the early stage.

Thus BCD07056 could be seen as a molecular chaperone which prevents aggregation during thermal unfolding by limiting the number of protein molecules engaged in the denaturation process that leads to aggregation. The way this molecule acts could be by (a) stabilizing the unfolded state preferentially over the intermediate state, (b) by destabilizing the intermediate, or (c) a combination of both.

Finally, the ability of β-cyclodextrins to accommodate solvent-exposed aromatic side chains into its cavity explains the wide range of cyclodextrin effects on different proteins: protection against degradation (if points of attack by proteases are masked by steric hindrances of cyclodextrins), alteration of biological function (if residues involved in function are masked by cyclodextrin) [3] and aggregation suppression (if residues responsible for aggregation are solvent accessible in the native or in a partially unfolded state of a protein). Thus the exact effects of cyclodextrins on a given protein always depend on its specific structure [3]. Consequently, these effects are difficult to predict and have to be investigated in each particular case. Our results show that the BCD07056 cyclodextrin derivative is a good additive to stabilize protein

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**Scheme 1.** Proposed denaturation Process of BlaPChBD in the absence of BCD07056. N denotes the native state; I and I′ the intermediate states; U and U′ the irreversibly and reversibly unfolded states respectively and A denotes the aggregated state. In the absence of BCD07056, the irreversible pathway leading to protein aggregation is favored. In the presence of the molecule the reversible pathway is mostly populated by the protein molecules.
without interfering with enzymatic and polysaccharide binding activities.

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