



Protein stabilization by cyclodextrins in the liquid and dried state[☆]

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ABSTRACT

Aggregation is arguably the biggest challenge for the development of stable formulations and robust manufacturing processes of therapeutic proteins. In search of novel excipients inhibiting protein aggregation, cyclodextrins and their derivatives have been under examination for use in parenteral protein products since more than 20 years and significant research work has been accomplished highlighting the great potential of cyclodextrins as stabilizers of therapeutic proteins.

Oftentimes, the potential of cyclodextrins to inhibit protein aggregation has been attributed to their capability to incorporate hydrophobic residues on aggregation-prone proteins or on their partially unfolded intermediates into the hydrophobic cavity. In addition, also other mechanisms besides or even instead of complex formation play a role in the stabilization mechanism, e.g. non-ionic surfactant-like effects.

In this review a comprehensive overview of the available research work on the beneficial use of cyclodextrins and their derivatives in protein formulations, liquid as well as dried, is provided. The mechanisms of stabilization against different kinds of stress conditions, such as thermal or surface-induced, are discussed in detail.

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1. Introduction

Maintaining stability throughout the life cycle of a therapeutic protein formulation is a very demanding task considering the multitude of degradation pathways proteins can undergo [1]. These degradation pathways are usually divided into either chemical or physical instability, although the distinction does not always apply. Whereas chemical degradation of a protein is understood as a change on the protein that involves the formation or the loss of covalent bonds [2], as e.g. encountered during deamidation [2–4], physical protein instability refers to unfolding and misfolding of the protein as well as to aggregation [4].

Protein aggregation is considered most problematic as it can occur at almost any stage of production, processing, storage and shipping of the protein drug. It can severely influence the pharmacokinetics as well as the safety of the protein drug, because the phenomenon of protein aggregation is closely linked to another challenge in protein formulation: unwanted immunogenicity [5]. For a break-down of immune-tolerance, the formulation and the occurrence of aggregates have been determined as influencing factors [6–8]. Considering the severe consequences of unwanted immunogenicity, such as loss of efficacy or formation of antibodies against endogenous proteins (as observed with patients receiving a certain erythropoietin-formulation [9]) it becomes evident that understanding and controlling protein aggregation is a major challenge for the development of safe and efficient protein formulations.

An important way of inhibiting protein aggregation is the addition of appropriate excipients to the formulations [4,10,11]. However, many of the commonly employed excipients suffer from certain shortcomings. For instance, non-ionic surfactants well stabilize therapeutic proteins against surface-induced aggregation, but may lead to increased aggregation and oxidation rates during quiescent long-term storage of the formulations [12,13].

Therefore there is a need for novel excipients for the inhibition of protein aggregation, either as alternative to currently used excipients or as complement. Cyclodextrins (CDs) have many times been reported to be a very promising class of excipients for the inhibition of protein aggregation [6,14]. They have been demonstrated to increase the thermal stability of liquid protein formulations [15,16], counteract surface-induced aggregation of proteins [17,18] and were also used as stabilizers during spray- and freeze-drying of proteins as well as for the manufacturing of microparticles for the delivery of therapeutic proteins [19–22]. However, to date they have not yet been used in approved parenteral protein products and little systematic overview on the extensive scientific work dedicated to their potential use in protein formulations is available. Wide ranges of CD-concentrations for use in protein formulations are reported in literature indicating only limited rational use of these excipients so far. Most importantly, various differing assumptions and hypotheses for the underlying molecular mechanisms of protein stabilization by cyclodextrins are reported.

By this work we intend to give a systematic overview on the use of CDs in protein formulations. Special attention will be dedicated to the underlying mechanisms of stabilization with the ultimate goal of contributing to a rational use of cyclodextrins in protein formulations.

2. Cyclodextrins for use in parenteral drugs

Cyclodextrins are applied for a multitude of purposes in the pharmaceutical field, e.g. to enhance solubility or bioavailability of small molecules or to mask bitter taste of certain drugs for oral use. For a general overview on pharmaceutical applications of CDs the reader is referred to one of the many excellent review articles and book chapters on the subject [14,23–29]. This work focuses on the application of CDs in protein formulations, i.e. on the role of cyclodextrins as excipients for a parenteral route of application. Therefore, first a brief overview on physicochemical as well as on toxicological properties of CDs is given, underlining the potential of CDs to be applied for parenteral administration.

2.1. Structure and physicochemical properties of cyclodextrins and their derivatives

Cyclodextrins (CDs) consist of three major classes of industrially produced, cyclic oligosaccharides, α -, β - and γ -CDs. The three native

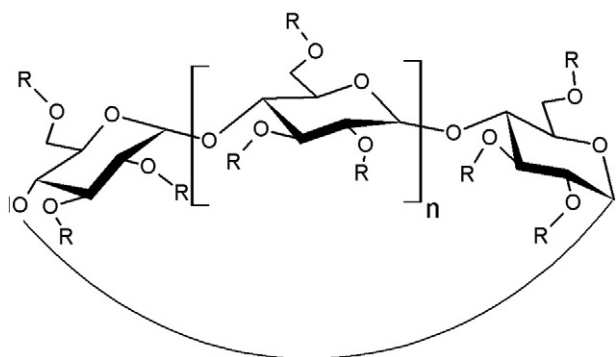


Fig. 1. General structure of cyclodextrins. For α -CD $n = 4$, for β -CD $n = 5$ and for γ -CD $n = 6$.

Table 1
Key characteristics of native CDs. Adapted from [24].

CD	α -CD	β -CD	γ -CD
Number of glucopyranose units	6	7	8
Molecular weight (anhydrous) [g mol ⁻¹]	972.85	1134.99	1297.14
Solubility per dm ³ H ₂ O at 298.2 K [g]	14.5	1.85	23.2
Annular diameter from the C (5) hydrogens [Å]	4.7	6.0	7.5
Annular volume [Å ³]	174	262	472

Table 2
Selection of CD-derivatives that are frequently used for pharmaceutical purposes.

Cyclodextrin	Abbreviation	Moiety (R)	Used in approved parenteral products
α -cyclodextrin	α -CD	H	Yes
β -cyclodextrin	β -CD	H	No
γ -cyclodextrin	γ -CD	H	No
Carboxymethyl- β -cyclodextrin	CM β CD	CH ₂ –COOH or H	No
Carboxymethyl-ethyl- β -cyclodextrin	CME β CD	CH ₂ –COOH, CH ₂ –CH ₃ or H	No
Diethyl- β -cyclodextrin	DE β CD	CH ₂ –CH ₃ or H	No
Dimethyl- β -cyclodextrin	DM β CD	CH ₃ or H	No
Glucosyl- β -cyclodextrin	G1 β CD	Glucosyl or H	No
Hydroxybutenyl- β -cyclodextrin	HB β CD	CH ₂ –CH–(CH–CH ₂)–OH or H	No
Hydroxyethyl- β -cyclodextrin	HE β CD	CH ₂ –CH ₂ –OH or H	No
Hydroxypropyl- β -cyclodextrin	HP β CD	CH ₂ –CHOH–CH ₃ or H	Yes
Hydroxypropyl- γ -cyclodextrin	HP γ CD	CH ₂ –CHOH–CH ₃ or H	No
Maltosyl- β -cyclodextrin	G2 β CD	Maltosyl or H	No
Methyl- β -cyclodextrin	M β CD	CH ₃ or H	No
Random methyl- β -cyclodextrin	RM β CD	CH ₃ or H	No
Sulfobutylether- β -cyclodextrin	SBE β CD	(CH ₂ –) ₄ –SO ₃ Na or H	Yes

CDs are crystalline, homogeneous, nonhygroscopic substances, which have a torus-like shaped structure. Since CDs are manufactured from starch they are built up from α -glucopyranose units. The enzyme forming CD is glucosyltransferase, which splits starch chains and rearranges molecules [25]. The system of nomenclature of CDs is based on the number of glucose residues in their structure. α -Cyclodextrin comprises six glucopyranose units, β -CD is a heptamer and γ -CD is constituted of eight glucose molecules. Fig. 1 shows a schematic representation of the general CD-structure.

So-called branched CDs result when a section of the amylopectin molecule containing a branching point is incorporated into the cyclic structure thereby attaching one or two glucosyl or maltosyl side chains to the ring. A number of papers indicate that branched cyclodextrins have remarkable potential for protein drug stabilization and delivery [18,30].

Crystal structures reveal that α -, β - and γ -CDs have an annular structure with a wide and a narrow hydrophilic end. The narrow end is defined by O(2)H and O(3)H secondary hydroxyl groups and the wide end is marked by O(6)H primary hydroxyl groups. The hydrophobic cavity consists of H(3), H(5) and H(6) hydrogens and O(4) ether oxygens. The glucose molecules in the ring structure are arranged rather rigidly in a ⁴C₁ chair conformation. Using neutron diffraction it could be demonstrated that the CD-structure is stabilized in the solid state by intramolecular hydrogen bonding between the secondary hydroxyl groups of neighbored glucose units [24]. Especially in β -CD, a complete secondary belt is formed by these hydrogen bonds. This in turn explains the remarkably low solubility of β -CD in water compared to α -CD (belt is incomplete) and γ -CD (more flexible, non-coplanar structure) [24]. Due to its low aqueous solubility native β -CD is rarely used in parenteral formulations.

The water solubility (w/w) of the three parent CDs at ambient conditions varies nonlinearly from approximately 13% to 2% to 26% for α -CD, β -CD and γ -CD, respectively [14]. Any substitution of the intramolecular hydrogen bond forming hydroxyl groups leads to a very clear increase of aqueous solubility even if the substitutes consist of hydrophobic residues such as methoxy and ethoxy functions because hydrogen bond formation of the unsubstituted hydroxyl groups with surrounding water molecules will be possible [25,27]. Furthermore derivatization not only improves solubility but it can also reduce the toxicological potential and hemolytic activity of many CDs, especially of β -CD [26,31,32], as described in more detail in the following paragraph. Table 1 summarizes important characteristics of the three parent CDs and some selected derivatives of relevance as excipients for parenteral administration.

Because of its cavity diameter dimensions and its good inclusion properties for aromatic rings (see below) β -CD-derivatives have gained a lot of attention. An important group of derivatives is that

of hydroxyalkylated CDs, such as hydroxyethyl-, 2-hydroxypropyl- and 3-hydroxypropyl-CDs [25]. For 2-hydroxypropyl- β -CD (HP β CD) both a European Pharmacopoeial and a United States Pharmacopoeial monograph exists specifying a molar substitution between 0.4 and 1.5 [33]. It is also specified that not more than 1.5% unsubstituted β -CD should be present [33]. HP β CD is one of two β -CD derivatives that have been used in approved parenteral products so far.

Another commonly applied derivatization is methylation. Especially randomly methylated β -CD has been closely examined for applications, as it provides relatively good biocompatibility as well as useful high complexation efficiency [14]. Sulfobutylether-derivatives belong to the group of CDs that has most recently been applied in FDA-approved parenteral products, notably the 2-sulfobutylether- β -CD (SBE β CD) which is contained in several commercially available preparations [14]. Table 2 gives an overview on some of the pharmaceutically relevant chemical derivatives of β -CD.

2.2. Parenteral biocompatibility of cyclodextrins

Safety is a major concern when new excipients for pharmaceutical formulations are introduced. Therefore also CDs need to exhibit good biocompatibility in order to be pharmaceutically considered. In the case of CDs toxicity is strongly dependent on the route of administration [26,32,34,35]. This review focuses on CDs and their role as excipients in protein formulations and therefore only parenteral biocompatibility is summarized in this paragraph.

The first important aspect of cyclodextrin toxicity is based on the extraction of membrane components from cells. Studies with CDs and isolated erythrocytes showed that CDs are capable of interacting with components of biomembranes [29,36]. The hemolytic activity was found to be in the order β -CD > α -CD > γ -CD reflecting the different solubilization rates of membrane components by each parent cyclodextrin [36,37]. The extraction of membrane cholesterol by CDs results in an increased membrane fluidity, which then induces membrane invagination ultimately leading to lysis of the cell at high doses of CDs. Besides cholesterol, CDs can also extract phospholipids. For β -CD it was even shown that it can remove proteins from erythrocyte membranes [26]. The mechanism of interaction with membranes is different from the one that can be observed with surfactants because CDs do not incorporate into the lipid bilayer but form new lipid containing compartments outside the membrane [26,30].

For parenteral administration toxicity on the kidneys is the most critical constraint. For α -CDs and β -CDs nephrotoxicity is manifested through alterations in vacuolar organelles of the proximal tubule and further cellular changes that are irreversible and ultimately toxic to the cells [26,38]. For β -CD additional toxicity arises from its low aqueous solubility, which leads to microcrystalline precipitation in the kidney. Furthermore,

the complexes formed by β -CD and cholesterol can accumulate in the kidney and produce renal tubule damage [39]. The LD₅₀ values of α -, β - and γ -CDs upon intravenous administration are approximately 1.0 g/kg, 0.79 g/kg and more than 4.0 g/kg, respectively.

Many of the described problems can be attenuated by functionalizing the parent CDs, e.g. by obtaining highly water soluble, amorphous hydroxyalkylether-CDs [14]. As described earlier, improving aqueous solubility can be achieved by almost any substitution on the native CD-molecules [25]. However, an increase in solubility does not necessarily also solve systemic toxicity problems. For example, the improved solubility of methylated CDs did not lead to reduced toxicity [26]. On the other hand, conversion of crystalline β -CDs into amorphous HP β CD by hydroxypropylation yielded highly water soluble molecules with very low systemic toxicity [32]. Furthermore, also sulfoalkylethers of β -CDs are reported to be well tolerated [40]. SBE β CD can be found in FDA-approved products as well as HP β CD [41] and both CD-derivatives have also gained most attention as potential excipients for protein formulations.

The toxicologic and pharmacologic effects of HP β CD have been extensively studied [32]. For instance, it was shown that a single intravenous dose of 10 g/kg of 50% (w/v) HP β CD was not lethal to cynomolgus monkeys [31] and in another study it was demonstrated that up to 1 g/kg of 20% w/v HP β CD did not cause premature deaths nor any other adverse clinical signs [32]. Subchronic studies were conducted in rats and dogs, for three months each, and doses as high as 50, 100 or 400 mg/kg/day were administered. In the 100 mg/kg/day group only minor histological changes such as granular kidney tubular cells were found whereas in the 400 mg/kg group these symptoms were more pronounced [42]. The main target organs, which revealed histopathological changes, were the kidney, liver, lungs and spleen. In an intravenous dosing study on human patients single doses of up to 3 g were determined to be well tolerated by all patients and kidney functions were not affected [43]. Altogether it can be concluded that the parenteral application of HP β CD is toxicologically safe [32]. This judgment is also reflected by the fact that HP β CD is contained in commercially available parenteral products, namely for the formulation of the antifungal agent itraconazol (Sporanox®) and also in parenteral formulations of Mitomycin C (MitoExtra®).

Another cyclodextrin derivative that is currently being used in commercially available solutions for infusion as solubilizing agent is sulfobutylether- β -cyclodextrin (SBE β CD). It is contained in intravenous voriconazole (Vfend®), in i.m. formulations of ziprasidone (Geodon®) and aripiprazole (Abilify®). Unlike other CD-derivatives SBE β CD is postulated to remain in an ionized state after glomerular filtration and thus not to undergo significant tubular reabsorption. By preventing reabsorption and concentration in the kidney, SBE β CD was designed to attenuate harm to the kidney [34]. In animal models SBE β CD was found to be essentially free of toxicological activity up to doses as high as 1500 mg/kg in dogs. Also in humans SBE β CD is generally well tolerated upon intravenous administration. Nonetheless, patients with a creatinine clearance <50 ml/min are advised to refrain from using intravenous voriconazole in order to prevent further renal dysfunction. This advice is further supported by two recent studies [44,45].

Intravenously administered CDs are rapidly eliminated from systemic circulation. Upon parenteral administration CDs are mainly excreted via the kidneys in an unmetabolized form [26]. Furthermore no deep compartment storage occurs and the V_{ss} is therefore comparable to the extra cellular fluid volume. In addition, it can be observed that the plasma clearance rates are in the same order of magnitude as for inulin which is known to be eliminated at the rate of glomerular filtration (about 110–130 ml/min in humans) [26].

3. Cyclodextrins as excipients for protein formulations

A number of drug-CD complexes have already been marketed for parenteral use. However, these products exclusively contain low-

molecular-weight drugs [14,46]. In the following section results of studies examining the potential use of cyclodextrins in liquid and dried protein formulations are discussed. Mainly due to their ability to bind to solvent-exposed hydrophobic residues CDs have been found to suppress aggregation of several therapeutically relevant proteins [47]. In turn this can also lead to destabilization of the unfolded state [48,49]. Further mechanisms of protein aggregation inhibition by cyclodextrins such as competition at the air–water interface are also discussed [17,18,50].

In Section 3.4 the effects of CDs on the stabilization of freeze-dried and spray-dried formulations as well as underlying mechanisms of stabilization are discussed.

CDs' ability to suppress aggregation has also been exploited for the use in simple artificial chaperone systems based on the ability of CDs to interact with denatured/aggregated proteins in a way that allows for natural refolding. These artificial chaperone systems frequently comprise a combination of a detergent and a CD [51–54].

3.1. Protein aggregation mechanisms in liquid protein formulations that can be affected by cyclodextrins

Protein aggregation can occur at almost any stage of manufacturing, processing, storage, shipment and administration to the patient. Generally the protein aggregation behavior in bulk solution is different from protein aggregation that involves adsorption to a bulk surface [55]. The influence of cyclodextrins on protein aggregation in bulk solution as compared to aggregation at interfaces is therefore discussed separately in the following sections. For detailed information on protein aggregation, its triggering factors and underlying mechanisms the reader is referred to one of the many excellent reviews on this subject [3,4,6,10,56].

The most common idea of how protein aggregation in solution proceeds is that partially unfolded states (also referred to as molten globule state or “A” states if the protein is acid-denatured) with reduced (but still substantial) secondary structure and clearly reduced tertiary structure expose hydrophobic surfaces and subsequently aggregate [57]. In order to suppress aggregation in bulk solution, it is necessary to maximize two physical protein properties: conformational and colloidal stability. When influencing protein aggregation cyclodextrins can potentially be expected to have an impact on both of these properties.

Increasing conformational stability means that the population of highly aggregation-prone partially unfolded intermediates is kept as low as possible. Conformational stability can be increased by selecting favorable solution conditions. An important factor leading to favorable solution conditions is an appropriate solution pH. Many proteins tend to slightly unfold on a tertiary structural level when acidic conditions are chosen, such as IgG-antibodies or rh-GH, two proteins that have also been examined in the presence of CDs [18,50,58].

Another important factor that compromises conformational stability of proteins and that therefore accelerates aggregation in bulk solution is temperature. High temperatures perturb the native protein conformation to a degree that accelerates aggregation [10]. Often aggregation starts well below the temperature that is experimentally determined as the melting temperature of the protein (the temperature at which 50% of protein molecules are unfolded during a thermal transition [59]), validating the assumption that aggregates are not formed from fully unfolded monomers but that a certain fraction of partially unfolded monomer is sufficient to promote aggregation [60]. Although accelerated stability studies at elevated temperatures were often found to inadequately represent protein storage stability at real-time [55,61,62], a number of examinations on the effect of CDs on protein aggregation were also carried out at high temperatures [15–17,63,64].

Ligand binding can also influence conformational stability. This is reflected by the Wyman linkage function, which states that preferential binding of ligands to the native state of a protein is expected to

shift the folding equilibrium towards a larger population of native protein molecules. Consequently the protein's propensity to aggregate will be reduced [62,65,66]. In contrast, preferential interaction with the unfolded or partially unfolded state of a protein will result in a decrease of the thermal stability of the protein, e.g. as observed with the preservative benzyl alcohol when binding to interleukin 1 or rh-GCSF [67,68]. These aspects of ligand binding are of special importance when discussing cyclodextrin–protein interaction and the consequences on protein stability: CDs are also reported to preferentially bind to the unfolded state of proteins [14], thereby inhibiting protein aggregation but potentially also influencing conformational stability of the proteins studied. Investigations on CD–protein interaction are discussed in more detail in the section below.

A global measure taking into accounts all sorts of intermolecular interactions between the protein molecules (van der Waals, electrostatic, hard-sphere) is given by the so-called colloidal stability of proteins, as expressed by the second virial coefficient (B_{22}). Although little work has so far been dedicated on the impact of CDs on colloidal protein stability [15], this aspect of protein aggregation should also be kept in mind when discussing the influence of CDs on protein aggregation.

In comparison to aggregation in bulk solution, the situation in the presence of large hydrophobic interfaces is very different, since other reaction pathways for protein aggregation are opened up. Especially when partial unfolding of a protein is the rate limiting step for aggregation, the presence of an interface can massively increase aggregation rates. The reason for this phenomenon is that proteins are amphiphilic molecules and this property leads to their strong tendency to accumulate at interfaces. Most proteins exhibit a remarkable adsorption to hydrophobic surfaces, the air–water interface not only being among the most hydrophobic but also most frequently encountered interfaces, e.g. during mechanical agitation and mixing, spray-drying or filtration. Other interfaces that therapeutic proteins are typically exposed to during their lifecycle may include the glass–water (in vials or syringes) or ice–water–interface (during freezing and thawing).

Agitation-induced aggregation has been reported for a variety of proteins [10,69–71,17,72–74], and it is a serious concern for the formulation of mAbs [75–80] and fusion proteins containing parts of immunoglobulins [81]. Aggregates formed by agitation have been determined to be very different in nature from aggregates of the same IgG-antibody formed during storage at elevated temperature [78]. The influence of CDs on agitation-induced aggregation was also studied [50]. Another protein for which the influence of CDs on agitation-induced aggregation was studied is rh-GH [18,58]. For recombinant human growth hormone (rh-GH) extensive investigations were carried out regarding the behavior after mechanical stressing

and exposure to the air–water–interface. Rh-GH was found to aggregate after vortexing or when being shaken in glass vials [71,74]. In addition investigations were carried out that concluded that shear forces alone cannot be made responsible for rh-GH-aggregation after mechanical stressing but that the presence of an air–water–interface is a necessary prerequisite [82]. This behavior was demonstrated by investigations using a rotor–stator–device and a nitrogen-bubbling-method [82]. Furthermore rh-GH tends to aggregate in the presence of other hydrophobic surfaces such as the ice–water interface [83]. Similar findings are reported for two further model proteins, which have also been studied in the presence of CDs, lysozyme and insulin [73,84].

3.2. Cyclodextrin protein interaction

The most common idea of how cyclodextrins inhibit protein aggregation is by binding to exposed hydrophobic residues on proteins. This binding is supposed to lead to a shielding of exposed surface hydrophobicity thereby blocking potential protein–protein interaction and inhibiting the formation of aggregates. In the following paragraphs evidence from literature on binding between cyclodextrins and proteins is discussed and molecular mechanisms as well as structural aspects of the interaction between cyclodextrins and the guest molecules are elucidated.

3.2.1. General molecular mechanism of complex formation between cyclodextrins and guest molecules

The central cavities of the CD-molecules are lined with skeletal carbons and etheral oxygens of the glucose residues, which render them lipophilic. It could be demonstrated that the polarity of the central cavity is comparable to the polarity of an aqueous ethanolic solution [25,27]. Therefore, a hydrophobic micro-environment is created in the interior while the cavity exterior of CDs is hydrophilic due to the presence of hydroxyl functions. This amphiphilic property of CDs is responsible for the aqueous solubility of the CD-molecules on the one hand and for the ability to encapsulate hydrophobic moieties on the other hand.

The incorporation of suitably sized drug molecules is the reason for most pharmaceutical applications of CDs [24]. During the drug–CD complexation process, no covalent bonds are formed or broken. In an aqueous solution there is a constant equilibrium between molecules that are included in the hydrophobic interior of the host CD and free molecules [14]. During the inclusion process either the entire guest molecule may be included or – as it is the case for proteins – only a certain part of the guest molecule is incorporated into the

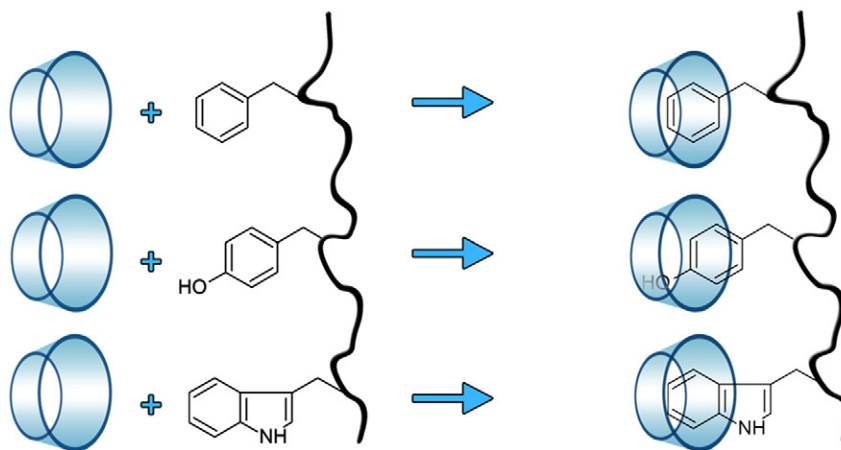


Fig. 2. Schematic representation of the proposed mechanism of cyclodextrin complexation of the amino acid residues of Phe, Tyr and Trp connected to the protein backbone (black line) according to Matsubara et al. [103] and Davis et al. [14]. The cavity of β -CD allows for the best fit of aromatic amino acids. Note that the scale of the figure does not represent actual molecule sizes.

hydrophobic cavity. The included molecules are normally oriented in the host in a position that allows for maximum contact between the hydrophobic part of the guest and the apolar CD cavity, thereby leaving as much as possible of the hydrophilic part of the guest molecule at the outer face of the complex [24]. That way maximum contact with both the solvent and the hydroxyl groups of the host-CD is ensured.

A number of different thermodynamic effects have been used to explain the complex formation. Extensive work has been dedicated on elucidating the thermodynamic driving forces of complex formation [85–88]. In most cases a rather large negative ΔH and a ΔS that can be either positive or negative are observed upon complex formation [27]. It was therefore concluded that “classical” hydrophobic interactions, which are associated with a positive ΔH and also a large positive ΔS (i.e. an entropy driven reaction), can be excluded as single driving force for complex formation. Some early investigations indicate that van der Waals forces are involved in the complexation process [27,89,90]. Other authors report hydrogen bonding as important factor [91,92]. A term named “compensation” is used to describe the fact that for many inclusion complexes between drugs and CDs a linear relationship between ΔH and ΔS is observed [27,88]. This observation is often taken as a hint that the release of enthalpy-rich water from the CD-cavity is the major driving force of the complexation process [93,94]. In addition, other effects like a release of ring-strain play a role as well [27,91].

3.2.2. Cyclodextrin–protein interaction: structural aspects

Several studies have been dedicated to elucidating the structural basis of binding between CDs and proteins. Almost all studies have identified aromatic amino acid residues as the main structural element interacting with CDs. Notably β -CD-derivatives with a cavity diameter that allows a fit of Phe, Tyr, His and Trp into the hydrophobic moiety exhibit this kind of interaction [47,58,95–100]. Fig. 2 outlines the proposed mechanism of interaction of cyclodextrins with aromatic amino acids residues. The interaction of α - and γ -CD with aromatic groups is reported to be much less effective due to steric reasons: the phenyl-ring can be tightly and deeply included into the β -CD cavity whereas α - and γ -CD lead to a shallow and loose or deep and loose inclusion, respectively [101]. However, according to Otzen et al., interaction should not generally be limited to aromatic amino acids [58]. Linear chains, for example of Ile, also allow a good fit into the α -CD cavity. Binding affinities of aliphatic amino acids towards β -CDs are several fold lower than binding affinities of aromatic amino acids towards β -CDs [58]. On the other hand also γ -cyclodextrin was reported to well interact with hydrophobic amino acid residues on glucagon, a polypeptide hormone [102]. In the studies on protein–CD interaction, a wide range of analytical techniques to identify sites and structural elements of interaction has been applied. Generally NMR studies turned out to be most informative.

A detailed study on protein–CD interaction was published by Otzen et al. [58]. In this study the interaction between human growth hormone (rh-GH) and different α -, β - and γ -CD derivatives was analyzed. Rh-GH seems to have a particularly pronounced tendency to interact with CDs, since it was also used as a model protein for the investigation of CD–protein interaction by other authors [18]. Even in its native state rh-GH exposes a considerable amount of aromatic amino acids making it prone to misfolding and aggregation on the one hand but also accessible for interaction with CDs. Employing NMR it was found that interaction between β -CD and rh-GH involves the H-3 and H-5 on the interior of the hydrophobic CD-cavity and Phe- and Tyr-residues on rh-GH. However, it was not possible to assign these interactions to specific amino acids on the molecule. Furthermore it was reported, that more homogeneous CD-products like maltosyl- β -CD with substitution only at the narrow CD-rim allow better access of hydrophobic amino-acid residues to the cavity than CD-products with randomly distributed substituents, supporting the hypothesis that complexation between amino acids and CDs proceeds via the wide rim of the cavity.

In a further study NMR spectroscopy was also chosen for the investigations because it is capable of providing atomic level information about complex supramolecular systems [47]. Circular dichroism in contrast was not sensitive enough to measure the rather weak interactions between rh-GH and β -CD. It was concluded that solvent accessibility of hydrophobic amino acid residues is a necessary but not compelling condition for the occurrence of CD–protein binding. In their study Aachmann et al. detected a multitude of interactions between aromatic residues (e.g. Phe) in an insulin-mutant and β -CD, but no interactions at all between aliphatic residues and β -CD [47]. Four interaction sites on the monomeric and one per monomeric subunit on the dimeric form of insulin were detected. Furthermore, the same study proved that for the interaction of two other proteins (C12 and S6) with β -CD also aromatic residues were responsible (Phe, Tyr). In contrast a fourth model protein, ubiquitin, could not be complexed at all, which is probably due to the fact that the water-accessible surface areas of the aromatic side-chains of ubiquitin are low [47].

NMR studies were also carried out to investigate the interaction of Alzheimer β -amyloid peptide (12–28) with β -CD [104]. It was found that the reason for β -CD to inhibit the conformational change of A β (12–28) from random coil to β -sheet in solution was the interaction of β -CD with A β (12–28). NOESY spectra exhibited cross peaks between H-3 and H-5 of the β -CD cavity and the aromatic protons of Phe19 and Phe20 indicating that the aromatic rings of Phe19 and Phe20 are inserted into the inner ring of the β -CD from the broad side. Interestingly there was also evidence for the insertion of the isopropyl-residues of Val118 into the β -CD cavity. No interaction at all was detected between of A β (12–28) and α - or γ -CDs. These CDs also failed to inhibit the aggregation of A β (12–28). NMR was as well employed to investigate the interaction between bovine serum

Table 3

Binding affinities of different β -CD-derivatives to therapeutic proteins. The global dissociation constants are listed if not indicated otherwise.

CD-derivative	Protein/peptide	K_d [mol/L]	Analytical method of determination	Reference
HP β CD	Rh-GH	$4.6 \cdot 10^{-3}$	Fluorescence titration	Otzen et al. [58]
M β CD	Insulin at 55 °C	$2.1 \cdot 10^{-3}$	ESI-MS and ^1H NMR	Dotsikas and Loukas [95]
HP β CD	Trp-Gly	$2.0 \cdot 10^{-2}$	Competitive spectrophotometry	Horsky and Pitha [96]
β -CD	Aspartame	$6.7 \cdot 10^{-3}$	Competitive spectrophotometry	Horsky and Pitha [96]
HP β CD	Melittin	$5 \cdot 10^{-2}$	Fluorescence (time-resolved and steady-state)	Khajepour et al. [97]
HP β CD	[D-Trp ⁶ , Des-Gly ¹⁰] LHRH	$8 \cdot 10^{-3}$	Isothermal titration calorimetry	Koushik et al. [98]
M β CD	Insulin	$K_{d1} = 5 \cdot 10^{-2}$ $K_{d2} = 1.5 \cdot 10^{-1}$	Dilution microcalorimetry	Lovatt et al. [112]
G ₂ β CyD (branched)	Rh-GH	$5.8 \cdot 10^{-3}$	Fluorescence method	Tavornvipas et al. [18]
HP β CD	Rh-GH	$1.3 \cdot 10^{-2}$	Fluorescence method	Tavornvipas et al. [18]
SBE β CD	Rh-GCSF	$9.28 \cdot 10^{-3}$	Surface plasmon resonance spectroscopy	Serno [113]
SBE β CD	IgG	$1.47 \cdot 10^{-3}$	Surface plasmon resonance spectroscopy	Serno [113]
β -CD	pH denatured chymotrypsin inhibitor 2	$18.9 \cdot 10^{-3}$	Stopped-flow fluorescence	Aachmann et al. [47]

Table 4

Summary of effects of different CD-derivatives on protein aggregation.

Protein	CDs	Molar ratio/ concentrations of CD and protein	Accelerated stability test	Effect on inhibition of aggregation	References
Lysozyme	12 different CD-derivatives	10–40	Chemical denaturation with GdmHCl	Branched β -CDs and DM β CD most effective	Tavornvipas et al. [16]
bFGF	9 different CDs	>10,000	Chemical denaturation with GdmHCl	DM β CD most effective	Tavornvipas et al. [16]
Lysozyme	5 different CDs	666	Thermal denaturation by DSC	Branched β -CDs and DM β CD most effective	Tavornvipas et al. [16]
bFGF	6 different CDs	830	Thermal denaturation by DSC	SBE β CDs most effective – because protein charged?	Tavornvipas et al. [16]
bFGF	7 different CDs	850	Acid inactivation	SBE β CDs most effective – because protein charged?	Tavornvipas et al. [16]
Salmon calcitonin	β -CD and derivatives	5	Thermal challenge at 55 °C	Native CDs no effect, HP β CD and RM β CD increase stability	Sigurjonsdottir et al. [64]
Bovine carbonic anhydrase	16 derivatives of α -, β - and γ -CDs	1500	Denaturation in 6 M GdmHCl	Acetyl-CDs most effective,	Sharma, Sharma [123]
O-GH	HP β CD	0–400	pH = 7.4 (O-GH stable at pH = 11)	Clear solution obtained	Brewster et al. [20]
Bovine insulin	HP β CD	400	Long-term stability	Good prevention of precipitation	Brewster et al. [20]
Porcine Growth Hormone	HP β CD	0–400	Thermal (63 °C for 1 h)	Significant reduction of precipitation	Charman et al. [17]
Porcine Growth Hormone	HP β CD	0–400	Guanidine dilution	Ineffective	Charman et al. [17]
Porcine Growth Hormone	HP β CD	0–400	Interfacial denaturation (vortexing for 60 s)	Significant reduction of precipitation	Charman et al. [17]
Rh-GH	β -CD, HP β CD and various branch-ed CDs	233 (50 mM CD and 4.73 mg/ml protein)	Chemical denaturation (4.5 M GdmHCl)	Branched CDs significantly inhibit aggregation, better than α -, γ - and HP β CDs	Tavornvipas et al. [18]
Recombinant Rh-GH	β -CD, HP β CD and various branch-ed CDs	233 (50 mM CD and 4.73 mg/ml protein)	Thermal denaturation (DSC)	Branched CDs significantly inhibit aggregation, better than α -, γ - and HP β CDs	Tavornvipas et al. [18]
Rh-GH	β -CD, HP β CD and various branch-ed CDs	233 (50 mM CD and 4.73 mg/ml protein)	Interfacial denaturation (vortexing)	HP β CD superior to other CDs due to its surface activity	Tavornvipas et al. [18]
Rh-GH	α -, γ -CD, various β -CD derivatives	Up to 70 mM of CDs used	pH = 2.5; 1 M NaCl	HP β CD, Glucosyl- β -CD, SBE β CD many fold more effective than α - and γ -CD, sulfated CD and monoacetyl-CD not effective at all	Otzen et al. [58]
Recombinant mink and porcine growth hormone	α -, β - and γ -CDs, varying degrees of substitution	Up to 150 mM of CDs, Up to 4.9 mg/ml of protein (ratio up to 670)	Renaturation after urea unfolding	Increased renaturation yield, best results with HP β CD and M β CD; Increased	Bajorunaite et al.[63]

Mink growth hormone	HP β CD, M β CD, Acetyl- β -CD, γ -CD	0.2 mg/ml mGH, up to 45 mM CD (ratio 4950)	Incubation at 60 °C for 5 h	HP β CD and M β CD most effective	Bajorunaite et al. [63]
Clostridium difficile Toxoid A (ToxA)	17 different CD-derivatives (α -, β - and γ -derivatives with varying degrees of substitution)	ToxA concentration 0.2 mg/ml; parent CDs 5–10 mM and CD derivatives 100 mM; maximal ratio hence about 150,000	Temperature ramp with 15 °C/min from 10 °C–87.5 °C	Inhibition of aggregation by most CDs under most conditions; for SBE β CD results strongly pH-dependent	Samra et al. [15]
Fibroblast growth factor 10 (FGF-10)	17 different CD-derivatives (α -, β - and γ -derivatives with varying degrees of substitution)	LcrV concentration 0.2 mg/ml; parent CDs 5–10 mM and CD derivatives 100 mM; maximal ratio hence about 9500	Temperature ramp with 15 °C/min from 10 °C–87.5 °C	Good aggregation inhibition by all derivatives at pH 5.5 and 7.5; promotion of aggregation by SBE β CD at pH 3.5	Samra et al. [15]
<i>Yersinia pestis</i> low-calcium-response V or V antigen (LcrV)	17 different CD-derivatives (α -, β - and γ -derivatives with varying degrees of substitution)	LcrV concentration 0.2 mg/ml; parent CDs 5–10 mM and CD derivatives 100 mM; maximal ratio hence about 18,500	Temperature ramp with 15 °C/min from 10 °C–87.5 °C	Inhibition of aggregation by most CDs under most conditions; for SBE β CD results strongly pH-dependent; no clear correlation to conformational stability	Samra et al. [15]
A β 40 peptide	α - and β -CD and hydroxypropylated derivatives	A β 40 peptide concentration was 25 μ M and CD concentration 10 mM; max. ratio hence 400-fold	Quiescent incubation at 37 °C for 21 days	Promotion of A β 40 aggregation and formation of fibrillar structures	Wang et al. [127]
Glycogen phosphorylase b	HP β CD	0.3 mg/ml protein and 0–114.6 mM HP β CD; max. ratio 37,207-fold excess of HP β CD	Thermal inactivation by quiescent incubation at 48 °C	Acceleration of aggregation process	Eronina et al. [124]
Glyceraldehyde-3-phosphate dehydrogenase (GADPH)	HP β CD	0–76 mM HP β CD and 0.4 mg/ml GADPH; max. ratio is 27,142-fold	Thermal inactivation by quiescent incubation at 45 °C	Acceleration of GADPH aggregation	Maloletkina et al. [125]
Creatine kinase from rabbit skeletal muscle	HP β CD	0–76 mM HP β CD and 0.2 mg/ml creatine kinase; max. ratio is 16,340-fold	Thermal inactivation by quiescent incubation at 48 °C	Prolongation of lag-phase prior to aggregate formation	Maloletkina et al. [126]
β -casein	α -, β -, γ -CD and Hydroxy-ethyl- β -CD	Molar ratio of up to 10:1	Incubation at 31 °C in the presence of Calcium	Significant inhibition of β -casein aggregation by β -CD; less inhibition by α - and γ -CD	Lee and Fennma [107]
Aminoacylase	HP α CD, HP β CD and HP γ CD	Molar ratio of up to 75,000	Refolding after denaturation in 4 M GdmHCl	Inhibition of aggregation in concentration-dependent manner; HP β CD most effective	Kim et al. [128]

albumin (BSA) and β -cyclodextrin derivatives [105]. The detected interactions included hydrophobic interactions, hydrogen bonds and electrostatic interactions with all kinds of interactions having a significant influence on the protein signals in the NMR spectra.

In other studies more indirect data about the interaction between proteins and CDs was obtained. For example, it was observed that maltosyl- β -CD decelerated α -chymotrypsin-catalyzed hydrolysis of buserelin acetate by incorporating the accessible hydrophobic side chains of the enzyme in the CD cavity at the substrate-binding site [106]. In a further study Lee et al. reported that β -CD is able to reduce the aggregation propensity of β -casein [107]. Upon the addition of Phe the ability of β -CD to inhibit β -casein aggregation was significantly reduced. It was concluded that the formation of a β -CD-Phe complex inhibited the formation of a β -CD- β -casein complex, which can be regarded as an indirect proof for the CD-complexation with this globular protein.

For studying the interaction of cyclodextrins with peptides containing aromatic amino acids Horsky and Pitha used competitive spectrophotometry [96]. In their study oligopeptides containing aromatic amino acids served as models for unfolded protein structures and it could be demonstrated that the affinity of interaction between CDs and the oligopeptides was at least as high as for the interaction of CDs and the isolated amino acids. An observed slight increase in affinity compared to studies with the isolated amino acids was explained by potential hydrogen bonding between the peptide backbone and secondary hydroxyls on the proteins. Yamamura et al. also used peptides to study the interaction between CDs and aromatic amino acid residues focusing on aspects of chiral recognition between host and guest molecules [108].

A further study [97] applied steady-state and time-resolved fluorescence spectroscopy as well as fluorescence polarization to study the interaction of a Trp-residue in melittin with HP β CD. Cryogenic measurements of the melittin fluorescence spectra showed Trp to be in a low water environment after addition of HP β CD. Therefore, interaction between the lone Trp residue and HP β CD is very probable. Again association of the polypeptide with the CD showed a dissociation constant (K_d) that is rather low and that lies within the range of the association of HP β CD with the single amino acid.

Finally another study [98] combines a number of different methods for the examination of the interaction of HP β CD with [D-Trp⁶, Des-Gly¹⁰] LHRH. Using UV and fluorescence spectroscopy a change in polarity of the environment of the chromophores occurred. Circular dichroism and ITC further supported the view that aromatic amino acids of deslorelin were included in the hydrophobic cavity of HP β CD.

In addition, a number of crystal structures of carbohydrate-binding proteins in complex with cyclodextrins can be obtained from the PDB database. For example the crystal structure of the enzyme cyclodextrin glycosyl transferase with β -CD can be found there [109]. Most of the interactions between CDs and carbohydrate binding proteins also seem to take place between aromatic amino acids and the hydrophobic interior of the CDs [47]. This was also confirmed by a study investigating the binding of β - and γ -CDs to glycogen phosphorylase b [110].

3.2.3. Stoichiometry and affinity of interaction

The unambiguous detection of binding between cyclodextrins and proteins is analytically challenging, since it is a comparably weak interaction, which is characterized by binding affinities in the mM-range (Table 3). So far CD-protein binding has been directly proven and quantified (as binding affinity) only for a few therapeutic proteins, e.g. insulin [95,111], rh-GH [16,58]. In these studies, also a clear and plausible relation between binding of cyclodextrins to proteins and aggregation inhibition by the same cyclodextrin derivatives could be demonstrated. In addition to the few interaction studies with therapeutic proteins, interaction of cyclodextrins with model peptides, which are easier to study due to their reduced complexity, thereby serving as model for larger proteins, was demonstrated [96–98]. Studies on model peptides included the di-peptide Trp-Gly

[96], Aspartame [96], Melittin [97] and [D-Trp⁶, Des-Gly¹⁰] LHRH [98]. The range of analytical techniques capable of monitoring protein-cyclodextrin interactions comprises direct fluorescence titration [58] (intrinsic steady-state fluorescence) as well as fluorescence lifetime measurements [58,97], NMR-measurements [47,95,104], isothermal titration calorimetry [98], dilution microcalorimetry [112], electrospray-ionization mass spectrometry [95] competitive spectrophotometry [96] and surface-plasmon resonance spectroscopy [113,114].

Table 3 gives an overview on the binding affinities between different CD-derivatives and proteins as well as model peptides that have been determined in the different studies. The global dissociation constants are listed if not indicated otherwise. An overall high degree of consistency among the obtained binding affinities can be observed, independent of the analytical method of determination, the CD-derivatives or the proteins and model peptides tested. Binding affinities generally lie in the mM-range, meaning that the interaction between CDs and proteins is rather unspecific and weak. As suggested by Otzen et al. it could be exactly this character of the interaction that makes it weak and dynamic enough to allow the proteins to sample different intramolecular conformations while the potential intermolecular interaction is blocked [58]. Some binding affinities for cyclodextrin-protein interaction that are reported in literature are significantly higher than what is listed in Table 3, e.g. for the binding of a bacterial transporter protein to cyclodextrins [114]. However, the bacterial transporter protein under investigation was a protein that specifically binds to cyclodextrins thereby allowing *Bacillus subtilis* to utilize cyclodextrins as a carbon source. This type of binding represents quite a different situation than the rather unspecific interaction between therapeutic proteins and cyclodextrin-derivatives as formulation excipients.

In most studies no clear stoichiometry of the binding of CDs to proteins could be determined. In the cases of peptides containing only one aromatic amino acid residue, such as for melittin [97], the site and stoichiometry of interaction are obvious. However, for rh-GH matters are already more complicated. Electrospray ionization mass spectrometry (ESI-MS) studies could show that rh-GH has at least two binding sites for the interaction with maltosyl- β -CD [30]. Further two-dimensional NOESY-spectra also indicate that rh-GH provides at least two different sites of interaction with β -CD in the native state [58]. For steric reasons probably only a fraction of potential interaction sites is populated by CDs at a given point of time. Naturally in the denatured state (for instance in the acid-denatured state as demonstrated for rh-GH [58]) an even larger number of amino acids will be solvent accessible and therefore potentially be able to interact with cyclodextrins. A study of thermal unfolding of proteins using DSC and assuming identical and independent binding sites on the unfolded state of the proteins came to the result that lysozyme, RNase and ubiquitin should contain 12, 5.4 and 3.7 binding sites, respectively [48]. These estimates are rather consistent with the aromatic amino acid contents of the three proteins (lysozyme 6 Trp, 3 Tyr and 3 Phe; RNase 0 Trp, 6 Tyr, 3 Phe and ubiquitin 0 Trp, 1 Tyr, 2 Phe). For the binding between BSA and mono-substituted β -CDs a complex stoichiometry of 1:4 was determined [105] and for the interaction of between methyl- β -CD and β -casein equilibrium interaction concentration ratio (molar) of 1.34:1 was calculated [107].

For insulin Aachmann et al. have determined four sites of interaction on the monomeric form of insulin and one per subunit on the dimeric form of insulin [47]. In that study, it was not possible to determine single dissociation constants for interaction sites. The data of Aachmann et al. is at least in part consistent with data from thermodynamic studies [112] in which – by using calorimetric dilution – data it could be determined that the insulin monomer contains at least two binding sites.

Mass spectrometry can generally provide useful information about the stoichiometry of the cyclodextrin-protein binding reaction [111,115–117]. The technique is a valuable tool for the detection of noncovalent bio molecular complexes [118,119]. Notably electrospray ionization mass-spectrometry (ESI-MS) with its unique

capability to also preserve weaker noncovalent bonds upon transferring the complexes from the solution phase into the gas phase was shown to be useful for the characterization of non-covalent protein complexes [119]. However, care should be taken when trying to correlate these MS gas-phase results to the binding-situation in solution. There are reports that show that correlation is not necessarily given [118].

Summing up literature reports on the binding stoichiometry between CD-derivatives and proteins, it can be stated that in most cases a 1:1 binding stoichiometry is dominating, but that the formation of higher-order complexes was also observed and that proteins can provide several binding sites for CDs [30,111,113,115,120–122].

Thermodynamic studies often demonstrated a reduction of the thermal stability of proteins in the presence of CDs. For globular proteins this has first been demonstrated by Cooper et al. [48,49]. They found that the binding of CD-derivatives to exposed side chains destabilized the native folded form of globular proteins (lysozyme, RNase A, ubiquitin, phosphoglycerate kinase) as evidenced by a decrease of T_m observed in DSC-studies. This observation was confirmed later on for example by Tavornvipas et al. [16] who found that the addition of a variety of CD-derivatives to lysozyme formulations leads to a decrease in T_m . In a study investigating the influence of different CD-derivatives on thermal stability of an IgG monoclonal antibody no significant impact of non-ionic CD-derivatives on T_m of the IgG was observed [50]. However, the only ionic CD derivative tested in this study, SBE β CD, dramatically lowered the temperature of unfolding by more than 10 °C. As later investigations revealed, SBE β CD was the only CD-derivative to actually bind to the IgG [113]. Ionic attraction due to opposite net charges between the IgG and SBE β CD enhanced binding, but was not a necessary prerequisite for interaction. Another study on three different model proteins, *Clostridium difficile* Toxoid A (ToxA), fibroblast growth factor 10 (FGF-10) and *Yersinia pestis* low-calcium-response V or V antigen (LcrV) also came to the conclusion that in many cases ionic SBE β CD-derivatives lead to a clear decrease of thermal stability (in a pH-dependent manner) [15].

Interestingly, also increases in thermal protein stability (e.g. measured as an increase in the onset temperature of unfolding) in the presence of CDs were observed in literature. For example in an earlier study Tavornvipas et al. reported an increase in T_m upon the addition of branched CDs to formulations of rhGH [18].

In conclusion it can be stated that binding between CDs and proteins was proven in many cases. Detailed structural data about interaction sites and the stoichiometry of interaction is available. In most cases the observed binding affinities were rather low, with equilibrium dissociation constants in the mM range. In the majority of the reported cases, protein–CD interaction leads to a shift of the folding equilibrium towards the more unfolded form of the protein, i.e. a decrease in conformational protein stability results. However, in some cases also an increase in conformational protein stability resulted, presumably due to preferential binding of cyclodextrins to the native state of the protein. There are also a number of reports about proteins not interacting with CDs. Many authors suggest solvent exposure and therefore accessibility of hydrophobic amino acid residues as a prerequisite for CD–protein complexation and apparently this prerequisite is not fulfilled for all proteins. In summary, the mode of interaction between CDs and proteins seems to depend on physical and chemical properties of the investigated proteins, the cavity size and the type as well as degree of substitution of the CDs under investigation.

3.3. Cyclodextrins as inhibitors of protein aggregation

As the discussion on CD–protein interaction has shown, CDs can lead to a preferential stabilization of the unfolded state. On the other hand interaction with hydrophobic groups on protein oligomers can lead to dissociation of protein aggregates, notably when

the interaction occurs at sites in the protein–protein interface. For example, this behavior was observed for the enhanced dissociation of bovine insulin dimers in the presence of different cyclodextrins [49,112]. Furthermore, not only the dissociation of existing aggregates by CDs has been reported but also the inhibition of protein aggregate formation *a priori* is demonstrated in a number of cases. Examples are available for insulin [95], rh-GH [18,58] and several other proteins [64,123]. A veritable number of further studies examining the effect of different classes of CDs on protein aggregation can be found in literature. Table 4 gives a comprehensive overview on available studies and results.

When analyzing the influence of CDs on protein aggregation it becomes apparent that depending on the nature of the investigated proteins, CD-derivatives and stress conditions quite different effects are observed. As a result the studies suggest a variety of mechanistic explanations for the different effects on protein aggregation. The studies as listed in Table 4 can be grouped into several main categories.

3.3.1. Reduction of aggregation due to binding between CDs and protein

The most frequent outcome of the available studies is that CD-derivatives, notably β -CD-derivatives, are able to suppress protein aggregation in bulk solution [16–18,20,58,63,107,124]. Many of these studies are conducted under elevated temperature conditions thereby fostering aggregation [15–18,63,64,124–127], others also present data after long-term storage [20,113]. In further cases chemical denaturation and subsequent dilution are used as aggregation model to study the influence of different CD-derivatives [16–18,63,123,128]. In addition, unfavorable solution conditions were chosen to foster protein aggregation and study the influence of CD-derivatives [20,58,107]. In the majority of these studies, β -CD derivatives are identified as the most suitable CD-derivatives to inhibit protein aggregation. Very often binding between CDs and the proteins under investigation is either implicitly assumed as underlying mechanism of stabilization or the aggregation inhibition results can actually be correlated to data on direct interaction between CDs and proteins. For instance, in the study of Tavornvipas et al. [18] a correlation between the extent of reduction of aggregation and binding constants between CD-derivatives and rh-GH was found. In their study branched CDs turned out to be most efficient in the prevention of chemically and of thermally induced unfolding and these CD-derivatives also showed the highest stability constants of all CDs tested in the study. HP β CD, which proved useful only in the prevention of interfacial aggregation of rh-GH, showed a weaker stability constant and was determined to be less effective in reducing aggregation in most cases. For the stabilization against chemically and thermally induced aggregation, i.e. non-interfacially induced protein aggregation, by branched cyclodextrins, efficient binding was identified as a prerequisite for stabilization. Also Otzen et al. were clearly able to correlate binding between different CD-derivatives and rh-GH to aggregation inhibition [58]. For instance, HP β CD was demonstrated to bind to rh-GH with an affinity in the millimolar range and inhibited aggregation at pH 2.5 in the presence of salt.

3.3.2. Reduction of aggregation induced by exposure to the air–water interface

Different aggregation pathways in bulk solution and at interfaces could be the reason for CDs to exhibit a different stabilizing effect against aggregation induced by interfaces than against aggregation in bulk solution. There are some hints available in literature that CDs could inhibit protein aggregation in a manner similar to non-ionic surfactants, i.e. mainly by displacing proteins from the air–water interface and thereby protecting them from denaturation and subsequent aggregation. For once, a number of studies indicate that cyclodextrins, e.g. HP β CD, are actually surface-active, which is a necessary prerequisite for competition at the interface [17,18,131–133]. Surface activity of M β CD and HP β CD was reported to strongly depend on the degree of substitution of cyclodextrins [18,131,133]. For

Table 5
Overview of studies using CD-derivatives as excipients in freeze-drying (FD) of protein formulations.

Protein(s)	CDs	Further excipients used	Analytical methods used for characterization of protein stability	Key results	Proposed protein stabilizing mechanism by cyclodextrins	Reference
Invertase (0.064 mg/ml)	β -CD (10% w/v), β -CD-alginate (10%), β -CD + trehalose (1:1, 10%)	Alginate, dextran, trehalose	Invertase assay with sucrose	β -CD and β -CD-A inferior to trehalose during FD, β -CD + trehalose showed best results during storage over 25 h at 43% controlled humidity; inhibition of trehalose crystallization	Complexation of hydrophobic residues for β -CD and β -CD-A	Santagapita et al. [167]
CYP3A4 (0.4 μ M)	M β CD, HP β CD (different molar ratios) (50:1–600:1 (~0.003–0.034% w/v))	2 crown ethers, sucrose, trehalose, mannitol	CYP3A4-activity with testosterone	CDs inferior to sugars, slight effect at very low concentrations	Water replacement	Chefson et al. [168]
LDH (0.25 mg/ml)	HP β CD (0.005%, 0.02%, 0.1%)	Sucrose, trehalose, glucose, dextran, PEG, Ficoll, polysorbate 80, Brij 35	LDH assay, PAGE	CDs inferior to sugars but better than no excipient and polysorbate 80	Surface activity	Anchordoquy et al. [166]
LDH (2 μ g/ml)	HP β CD, M β CD, α -CD, β -CD, γ -CD (0.005 mM–100 mM)	PEG 3350, CHAPS*, sucrose fatty acid monoester, Polidocanol, SDS, glucose, trehalose, sodium glutamate	LDH assay	HP β CD and M β CD preserved enzymatic activity during FD at concentrations of 0.1 mM to 10 mM, no effect of parent CDs; HP β CD stabilization independent of phosphate-buffer concentration	Surface activity, interaction with hydrophobic residues	Izutsu et al. [172]
LDH (12.8 μ g/ml)	α -CD, HP α CD, β -CD, HP β CD, SBE β CD, M β CD, γ -CD, HP γ CD (1% w/w) and 0.0001% to 1% (w/w) for HP β CD	Glucose, maltose, trehalose, sucrose, raffinose (1% w/w)	LDH assay	HP α CD, HP β CD, M β CD, and HP γ CD superior to trehalose; other derivatives provide stability similar to trehalose and sucrose; increasing degree of substitution (DS) correlated with increase in relative activity	No clear statement: water replacement/amorphous glass forming/complexation	Iwai et al. [176]
TNF (0.25 mg/ml)	HP β CD (0.5% w/v) + mannitol 1.5%	PEG 6000, dextran, sucrose, trehalose	TNF assay (cell killing), SDS-PAGE	HP β CD + mannitol resulted in less dimer formation than lyophilization without excipient; bioactivity was preserved	Amorphous glass forming	Hora et al. [175]
Mouse IgG2a mAb (1.0 mg/ml)	HP β CD (5% w/v)	Sucrose, dextran	SDS-PAGE, SE-HPLC, ELISA, IEF	HP β CD comparable to sucrose and dextran after lyophilization but superior during storage at 56 °C; HP β CD inhibited a aggregate formation at 4 °C for 32 days.	Amorphous glass with high T _g , complexation	Ressing et al. [174]

BSA (0.1%), trypsin (0.1%)	α -CD (9.9% w/w)	Lactose, sucrose, mannitol, dextrin, Tween 80, some others	Trypsin assay, SEM, DSC, ESCA (electron spectroscopy for chemical analysis)	α -CD showed highest trypsin activities and low trypsin surface coverage but high BSA surface coverage after annealing; no correlation of surface coverage and activity	No clear statement; carbohydrate-like behavior (water replacement, amorphous glass forming)	Millqvist- Fureby et al. [169]
β -Galactosidase (0.1 mg/ml)	HP β CD, M β CD, α -CD, β -CD, γ -CD (0.01 mM–10 mM)	Glucose, trehalose, proline, Polidocanol, SDS, CHAPS*, sucrose fatty acid monoester, PEG 400, 3350	β -Galactosidase assay with galactopyranoside-derivative, SE-HPLC, SDS-PAGE,	HP β CD and M β CD showed stabilizing effects at concentrations above 0.1 mM, α -CD, β -CD and γ -CD not; HP β CD and DM- β -CD showed positive effect in reconstitution medium	Surface activity	Izutsu et al. [171]
IL-2 (0.8 mg/ml)	β -CD (5 mg/ml)	Mannitol, sucrose, trehalose, raffinose, stachyose	FTIR, turbidity, activity assay, SE-HPLC, SDS-PAGE	Stabilization by β -CD	High Tg of dried product and water replacement	Prestrelski et al. [177]
LDH (2 μ g/ml)	HP β CD 0.01 mg/ml–10 mg/ml (+ mannitol 400 mM/sucrose 200 mM)	CHAPS, PEG 400, 3000, 20,000, Polidocanol, Triton X-100, Brij 58, sodium cholate, sucrose monolaurate	LDH assay	HP β CD showed best stabilizing effect used alone and intermediate effects when used in combination with mannitol and sucrose	Surface activity, formation of an amorphous glass	Izutsu et al. [165]
IL-2 (1 mg/ml)	HP β CD (0–25% w/v) and 0.2%–2% in combination with sucrose (1%–2%, all w/w); β -CD (concentration not stated)	–	Visual inspection, turbidity, Western blot, centrifugation, 4D UV, bioactivity (HT-2 cell proliferation assay)	HP β CD reduced turbidity in concentrations of >0.5% in combination with sucrose (1%) during storage, no turbidity was observed with HP β CD but with β -CD, both in combination with sucrose (1–2%)	Complexation	Brewster et al. [178]
IL-2 (1 mg/ml)	HP β CD (0.5%) in combination with sucrose (2%)	Polysorbate 80, HSA, arginine, carnitine, betaine	SE-HPLC, RP-HPLC, Western blot, Fluorimetric light scattering, bioactivity (HT-2 cell proliferation assay), ELISA	Fully bioactive IL-2, negligible dimer formation but significant amount of oxidized IL-2 detected during storage	Unclear: hydrophobic/ hydrophilic character of HP β CD	Hora [179]

*: abbr. for (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate).

instance for HP β CD values between 69 mN/m and 52 mN/m are reported for degrees of substitution ranging from 2.5 to 11.3, respectively. In addition to cyclodextrins being reported as surface active there are also studies available that relate the inhibition of surface-induced aggregation by HP β CD to the surface-activity of the excipient [17,18,50]. The effectiveness of HP β CD in reducing interfacially induced precipitation of porcine growth hormone was ascribed to the surface activity of HP β CD and it was speculated that the mechanism was analogous to that proposed for the stabilization by polysorbate 20 [17]. In another study the proposed relationship between the interfacial stabilization of rh-GH by HP β CD and surface activity of HP β CD was substantiated by correlating increasing degrees of substitution of HP β CD (that translate into increasing surface activity) to reduced amounts of aggregates in vortexed rh-GH formulations [18]. Furthermore in studies on aggregation inhibition of an IgG it was also suggested that HP β CD could have stabilized the protein by competition at the air–water interface [50]. In these studies it was shown that HP β CD turns out to be a valuable alternative to non-ionic surfactants like polysorbate 80 for the stabilization of IgG-formulations against aggregation induced by the air–water interface. Also, it was suggested that disadvantages of non-ionic surfactants like reduced long-term storage stability in quiescent solution might not be encountered in the presence of HP β CD. However, so far detailed studies proving the mechanism of protein displacement from interfaces are lacking and there are results available pointing into the direction that the stabilizing mechanism of CDs at the air–water interface is different to that of non-ionic surfactants [113]. Surface-activity and the presence of CDs at the air–water interface seem to be a necessary prerequisite for stabilization, but nevertheless CDs are apparently unable to displace proteins from the interface but exert other stabilizing mechanisms.

3.3.3. Further mechanisms of stabilization

In some cases a reduction of protein aggregation was reported but could not necessarily be attributed to binding between CDs and the investigated proteins [15,16,50,113]. An obvious assumption when discussing the stabilizing mechanism of cyclodextrins on proteins could be to believe that cyclodextrins – since they are chemically classified as sugars – also act by preferential exclusion in a way similar to low molecular weight sugars and polyols. Sucrose is probably the most studied excipient of this group. The interaction between sucrose and the protein is thermodynamically unfavored because of strong repulsion between the protein backbone and sucrose [62,129]. Thus the greater the surface area of the protein, the more sucrose will be excluded from the protein surface. As during unfolding surface area increases, the amount of “negative binding” between sucrose and the protein increases. Consequently, in the presence of high amounts of sucrose, the native protein state is favored. For cyclodextrins this mechanism of stabilization was also discussed, especially at high concentrations of CDs employed [15,130]. However, the assumption has not been verified so far and remains to be further investigated.

3.3.4. Increased aggregation rates due to cyclodextrin addition

Cyclodextrin–protein interaction does not necessarily always translate into a reduction of aggregation. In several cases increased protein aggregation rates as a consequence of CD-addition were reported. Especially the use of SBE β CD was reported to trigger very different effects on protein aggregation. Whereas for instance aggregation of the positively charged bFGF after acid-induced denaturation was reported to be decreased by the addition of SBE β CD [16], there are other reports in which SBE β CD leads to an increase of aggregation rates of the proteins under investigation. This was the case for aggregation of an IgG after storage at elevated temperature [50]. In other cases, the effects of SBE β CD on aggregation were reported to be strongly pH-dependent [15]. This dependency is a clear indication that besides inclusion processes also ionic interaction between SBE β CD and proteins influences interaction and therefore aggregation rates. It was also speculated that

ionic SBE-CD-derivatives could have an impact on colloidal stability of protein solutions by shielding of charge–charge interactions.

However, detrimental effects of CDs on protein stability were not only published in the case of SBE β CD but also e.g. for HP β CD [124,125]. Aggregation of the oligomeric proteins glycogen phosphorylase b and glyceraldehyde-3-phosphate dehydrogenase (GADPH) was accelerated in the presence of HP β CD. In the studies, it was concluded that the acceleration of thermal denaturation by HP β CD is typical for oligomeric proteins for which denaturation proceeds by a dissociative mechanism.

3.3.5. No effect of cyclodextrins on protein stability

It has to be noted that although a wealth of studies proving the inhibition of protein aggregation by cyclodextrins are available, comparably few reports on therapeutic proteins of commercial relevance (at concentrations of therapeutic relevance) have been published. Rh-GH is the remarkable exception to this rule, but (as discussed before) the unusually high percentage of solvent-accessible aromatic amino acids renders it more prone for interaction with CDs than other therapeutic proteins. For the most important class of therapeutic proteins, monoclonal antibodies, few studies are available. The reason for this behavior could be that CDs are not particularly effective at inhibiting monoclonal antibody aggregation during quiescent storage in bulk solution, especially at the very high concentrations that are currently in the focus of protein formulation development [134–136]. There is data available pointing into the direction that some CDs have no or only marginal effect on IgG stability during quiescent (unagitated) storage [50,113]. Also for other proteins such as bovine serum albumin and lysozyme it has been published that the addition of CDs did not alter the stability of the investigated proteins [15].

In summary it seems that particular proteins with significant solvent-exposure of hydrophobic amino-acid residues are suited for stabilization by CDs. Rather hydrophilic proteins with little exposure of hydrophobicity remain unaffected by the presence of CDs. In contrast aggregation induced by protein exposure to interfaces follows different patterns: here CDs exert stabilizing effects that depend less on the structure of the protein but more on surface-activity and surfactant-like effects of the CD-derivatives under investigation.

How could CDs be used in a more rational way in protein formulations? Table 4 suggests that what might be useful for one therapeutic protein can be without any significant effect for another protein or even compromise protein stability. CD-derivatives inhibiting aggregation induced by a certain stress condition can be incapable of inhibiting aggregation induced by another stress condition. The absolute concentrations as well as molar ratios between CDs and proteins greatly vary. To solve this problem and in order to provide a rational for using CD-derivatives in protein formulation, Aachmann et al. suggested paying attention to highly solvent accessible exposed hydrophobic residues on the proteins, which could be an important but not compelling prerequisite for the interaction of proteins and CDs [47]. Another systematic approach would be to carefully choose appropriate CD-derivatives since the type of substitution is of great influence on the capability to inhibit aggregation (Table 4). A further way towards a more rational application of cyclodextrin-derivatives in protein formulation takes into account the stabilizing role that CDs are supposed to exert in the respective protein formulation: direct interaction and stabilization via blocking of hydrophobic interaction might only be possible in a certain limited number of cases whereas the use of CDs for the prevention of surface-induced aggregation could be a generally feasible property of CDs. In this regard, some CDs could be used as valuable alternative to non-ionic surfactants.

3.4. Cyclodextrins as excipients for freeze- and spray-drying of protein solutions

3.4.1. Stresses arising from freeze- and spray drying processes

In order to achieve acceptable shelf lives for protein drugs, drying is often applied [137,138]. In the pharmaceutical industry

Table 6
Overview of studies using cyclodextrins as excipients for spray-drying (SD) or supercritical fluid drying (SCFD) of protein formulations.

Protein(s) used	CD(s) used	Other excipients used	Analytical methods used for characterization of protein stability	Outcome of test	Proposed protein stabilizing mechanism of cyclodextrins	Author(s)
β -Galactosidase (4% w/v) Trypsin (0.02%, 0.1%, 0.5% w/w)	HP β CD (1% w/v) alone and in combination with sucrose 1% α -CD (9.5%–9.98% w/w)	Sucrose Lactose, sucrose, mannitol, dextrin, Tween 80	μ DSC, SE-HPLC, SDS-PAGE, galactosidase assay. ESCA, DSC, surface tension, trypsin assay	HP β CD superior to sucrose α -CD showed good results in residual activity and was best excipient in FD	Inhibition of denaturation at liquid/air interface No clear statement; carbohydrate-like behavior (water replacement, amorphous glass forming)	Branchu et al. [173] Millqvist-Fureby et al. [170]
rhGH (2 mg/ml)	M β CD (10, 100, 1000:1 molar ratio, 0.12%, 1.2% and 12% w/v), 100:1 M β CD also in combination with polysorbate 20 (0.01%), zinc (2:1 molar ratio) and lactose (4 mg/ml) HP β CD (80 mg/ml)	Polysorbate 20, lactose, zinc Trehalose (80 mg/ml)	SE-HPLC FTIR, CD, fluorescence, UV spectroscopy, SE-HPLC, SDS-PAGE	M β CD stabilizes in molar ratio of 1000:1 and 100:1 (SE-HPLC) and in combination with zinc, lactose and polysorbate 20 at 100:1 molar ratio HP β CD as efficient as trehalose	Surface activity	Jalalipour et al. [180] Jovanovic et al. [181]

freeze-drying, also called lyophilization, has become the most important drying technique for proteins due to the gentle nature of the freeze-drying process [137,138]. Besides lyophilization, spray-drying has also been intensively investigated, but so far it is not yet routinely used for the manufacture of commercial protein products.

Generally, freeze-drying is regarded as a three-step process starting with cooling and freezing, followed by primary drying and secondary drying. The freeze-drying process exerts several stress conditions on proteins [137,139,140]. Excipients can mitigate the stress conditions exerted on proteins [137,141].

Cold denaturation occurring during freezing of protein solutions is a possible stress factor. However, cold denaturation is reported to only contribute to a very low amount to the overall stresses encountered during lyophilization [142].

During cooling, phase separation occurs and ice crystallizes, causing the concentration of the remaining solutes to increase (freeze-concentration). The concentration of salts or sugars may increase up to 20-fold during freezing, which alters the quantitative solution composition and hence properties like ionic strength and pH, potentially negatively affecting protein stability [137,142,143].

In addition ice crystallization leads to exposure of proteins to a large ice–water interface, which may also lead to accelerated protein aggregation, because proteins are surface-active, amphiphilic molecules and may therefore adsorb to the interface and denature [142,144,59]. Dehydration itself is a further challenge for protein stability because the hydration shell surrounding the protein is lost [139]. The loss of the hydration shell may alter the native state of the protein and cause denaturation due to loss of charges and increased protein–protein hydrophobic interactions [137,145].

In contrast to freeze-drying, where the drying process is based on sublimation from the bulk solution, for spray-drying atomization of the liquid into droplets and evaporation of the solvent is the prevalent drying mechanism [139].

Comparably high temperatures are often encountered during spray-drying leading to potential denaturation of proteins [59]. Further possible degradation of proteins during spray-drying may also be caused by the large air/water interface which is generated during atomization.

3.4.2. General mechanisms of stabilization by excipients during freezing, drying and in the dried state

Excellent reviews have been published summarizing the stabilization of proteins during drying and in the dried state [137,141,146,147]. Commonly used excipients, which can counteract the aforementioned stresses and their mechanisms of stabilization, are presented briefly in the following section.

To prevent unfolding of proteins at high and low temperature stresses, disaccharides like sucrose or trehalose or polyols like sorbitol or mannitol are commonly employed, acting via the preferential hydration/exclusion mechanism [62]. In the field of freeze-drying, those excipients are employed to stabilize the protein drug during the freezing step and are referred to as cryoprotectants. Polymers, like dextran, polyvinylpyrrolidone and polyethylenglycol (PEG) are commonly used as cryoprotectants as well [147].

Unfolding of proteins at interfaces can be prevented by surfactants. Surfactants, primarily the non-ionic polysorbate 20 and 80 are frequently added to the bulk solution to inhibit unfolding of proteins at interfaces, for example at the air–liquid interface present during spray-drying and at the ice–water interface, occurring during the freezing step in freeze-drying [148–150]. As mentioned in a preceding paragraph, surfactants are known to compete with proteins for the adsorption to interfaces and hence reduce unfolding and subsequent aggregation at the interface [50,157].

To preserve the activity of proteins during dehydration, two commonly accepted hypotheses are discussed: the water replacement

[151–154] and the vitrification [155–159] hypotheses. Excipients stabilizing the protein during the drying stage and in the dried state are referred to as lyoprotectants [137].

During dehydration, the hydration shell surrounding the protein is lost, and the loss of hydrogen bonds can be compensated by excipients able to interact with the protein by hydrogen-bonding, thus serving as a water substitute. Disaccharides like sucrose and trehalose and polyols like sorbitol are commonly used lyoprotectants. By acting as a water substitute, the native protein structure is preserved and the protein is thermodynamically stabilized with an increase in free energy of the unfolded state [146,160]. Usually, direct interaction of the stabilizer and the protein and defined minimum ratios of stabilizer to protein are needed [161,162].

The vitrification (or glassy dynamics) hypotheses refer to a kinetic mechanism of stabilization in the dried state. A steady increase in viscosity of the amorphous phase, during the freezing and dehydration steps in freeze-drying or during the evaporation stage in spray-drying leads to the formation of an amorphous glass [163]. An adequate glass forming excipient needs to be present. Commonly used excipients are disaccharides and polymers like dextran and PEG [163]. In the rigid environment below the glass transition temperature, featuring a high viscosity, the protein is immobilized and hence kinetically stabilized. In contrast to the water replacement hypothesis, not a molar ratio of stabilizer to protein but a certain volume of stabilizer is required for dilution of the protein into the amorphous matrix [160].

3.4.3. Possible mechanisms of stabilization provided by cyclodextrins

Due to their particular structural properties, cyclodextrins are valuable excipients for drying of protein pharmaceuticals. A CD-derivative frequently used as stabilizer in freeze-drying and spray-drying of protein formulations is HP β CD, which in contrast to the parent α -, β - and γ -cyclodextrin shows surface activity at concentrations of 0.1% (w/v). [133]. This amphiphilic quality of HP β CD may stabilize proteins during the cooling and freezing step and upon rehydration of lyophilizates as well as during the atomization step in spray drying, possibly inhibiting protein unfolding and aggregation at interfaces, as discussed above.

Being carbohydrates CDs are in principle able to interact with proteins by hydrogen bonding and to fulfill the needs for water replacement during dehydration. However, it is known that especially the parent cyclodextrins form an intramolecular hydrogen bond ring [164]. Therefore, parent cyclodextrins should be less able to provide intermolecular hydrogen bonds to proteins and this mechanism of stabilization must be expected to be operative mainly for the substituted derivatives, especially hydroxypropylated derivatives.

In literature, the assignment of CDs to an assumed stabilizing mechanism is inconsistent. CDs are referred to as surfactants

[165,166], polymers [167] or sugars [168,169], indicating that the stabilization mechanism is not yet fully understood.

3.4.4. Use of cyclodextrins as excipients for drying of proteins and underlying mechanisms of stabilization

There are several reports on the use of cyclodextrins with different drying techniques. An overview of these studies is presented in Table 5 and Table 6. In the nineteen-nineties, several studies were performed using enzymes as model proteins assessing the stabilizing potential of CDs by determining their enzymatic activity after drying and reconstitution [165,169–173]. Besides those model proteins, also reports on drying of therapeutic proteins, such as a mouse IgG antibody [174], IL-2 and tumor necrosis factor (TNF) [175] in the presence of CD derivatives were published. Cyclodextrins were investigated in a wide range of concentrations, ranging from 0.0001% (w/v) up to 10% (w/v) [169,176]. From these numbers it can already be deduced that CDs presumably fulfilled very different roles in these formulations. The following chapter gives an overview on the available studies and elucidates the different molecular mechanisms by which CDs can provide stabilization during the drying of proteins as well as during storage of dried protein formulations.

3.4.5. Protein stabilization by cyclodextrins at low CD-concentrations – non-ionic surfactant-like behavior

Evidence for surfactant-like effects of cyclodextrins was found for freeze-dried LDH at a concentration of 0.25 mg/ml in the presence of HP β CD at different concentrations (0.005%, 0.02% and 0.1%) [166]. HP β CD showed a slightly positive effect on remaining enzyme activity after reconstitution of lyophilizates and in freeze-thawing experiments at concentrations of 0.1%, and it was found to be superior to polysorbate 80. However, enzymatic activity was much better preserved by employing glucose, trehalose or sucrose. Slightly different results were observed in another study with freeze-dried LDH at much lower concentrations (2 μ g/ml protein) [172]. HP β CD and M β CD turned out to be effective in preserving enzymatic activity. The protective effect of HP β CD, as measured by LDH activity, was most pronounced at concentrations above 1 mM (approx. 0.14% w/v). M β CD showed best stabilization at 0.2 mM (approx. 0.026% w/v), while the parent cyclodextrins as well as sucrose and trehalose showed almost no remaining LDH activity after lyophilization and reconstitution. A third study using LDH as model protein achieves similar results. A wide concentration range of HP β CD from 0.0001% to 1% (w/w) was investigated and could clearly show that HP β CD was able to preserve the enzymatic activity already at very low concentrations (0.01%) with a maximum stabilization between 0.1% and 1% [176].

In addition to the concentration-dependent stabilization by HP β CD, the degree of substitution (DS) of the CD-derivatives was also found to have a significant influence on recovery of LDH activity.

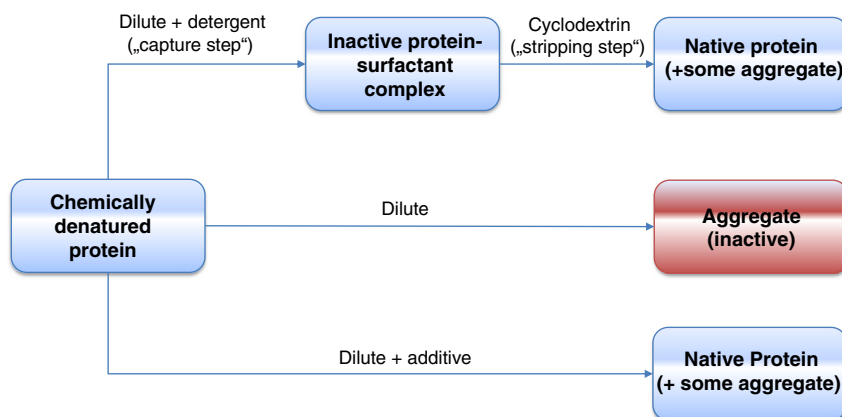


Fig. 3. Artificial refolding of denatured proteins. Adapted from [53].

The higher the degree of substitution, the better was the recovery of activity of LDH. It is known that an increase in the degree of substitution of the CD correlates with a decrease in surface tension of water – therefore the surfactant-like effects of CDs must be expected to become more pronounced with increasing DS [18,133].

HP β CD was also studied by Izutsu et al. with the same model protein, LDH, in a concentration of 2 μ g/ml as single excipient and in combination with 200 mM sucrose and 400 mM mannitol [165]. At concentrations of around 0.01% (w/v) and 0.1% (w/v), HP β CD showed best results when used alone and comparable results were obtained in combination with sucrose whereas the combination with mannitol resulted in slightly lower activity at the same concentrations. The authors state that the amphiphilic character of HP β CD and its ability to remain amorphous is protecting LDH during freeze-drying, which is in agreement with results obtained on another enzyme, β -galactosidase [171]. Parent cyclodextrins formed an amorphous glass, but failed to stabilize β -galactosidase during freeze-drying whereas M β CD and HP β CD were effective at maintaining the enzymatic activity at concentrations of 0.014% (w/v), indicating surfactant-like behavior of the CD-derivatives.

Further evidence for interfacial stabilization by CDs is given by a study on spray-dried β -galactosidase at a concentration of 40 mg/ml [173]. The enzyme was spray-dried along with 1% (w/v) HP β CD, a mixture of 1% (w/v) HP β CD and 1% (w/v) sucrose and sucrose alone. HP β CD as single excipient yields almost 100% remaining activity whereas sucrose alone even had a negative impact on the relative activity of the enzyme. The combination of HP β CD and sucrose was inferior to HP β CD alone. No alterations of the enzyme as compared to before drying were detected by DSC, SE-HPLC, SDS-PAGE and IEF patterns in all formulations. The authors speculate that the mechanism of protein degradation consisted of subtle changes in protein structure at the air–water interface and they concluded that HP β CD successfully protected β -galactosidase from surface denaturation. This was also observed for M β CD, which protected rh-GH during spray-drying at concentrations above 1.2% (w/v) [180]. The addition of polysorbate 20, lactose and zinc further reduced aggregate levels, as determined by SE-HPLC. The authors conclude that surfactant-like behavior of M β CD is protecting rh-GH from denaturation at the air–water interface.

However, there are also reports on amphiphilic cyclodextrin derivatives failing to stabilize proteins at low excipient concentrations. For instance, M β CD and HP β CD failed to stabilize the enzyme CYP3A4 [168]. Both derivatives showed no significant increase in activity recovery of CYP3A4 at very low protein concentrations and a 50:1 molar ratio excess of the CDs (approximately 0.003% (w/v)). Disaccharides like sucrose or trehalose performed better than both cyclodextrin derivatives; however, they were used at much higher molar ratios up to 30,000:1. This indicates that dehydration could have been the predominant stress for CYP3A4 and disaccharides are able to compensate for the loss of the hydration shell. The concentrations for the cyclodextrins employed in this study are probably too low to serve as sufficient lyoprotectants whereas their quality as surfactant-like excipients did not alter protein stability in this case.

3.4.6. Protein stabilization by cyclodextrins used at high concentrations – water replacement and vitrification

Besides their effect on stabilization at the air–water, ice–water and solid–void interface, cyclodextrins are also reported to stabilize by both water-replacement and vitrification. However, the clear attribution of the mechanism of stabilization to one particular effect might be difficult, since several modes of action often contribute to the protein stabilization observed. For the purpose of employing CDs as lyoprotectants, much higher weight ratios are needed compared to the use as surfactant like excipients; typical weight ratios of sugar to protein range from at least 1:1 to approx. 5:1 [141]. However, these ratios are greatly dependent on the type of protein. Concentrations of bulking agents

prior to freeze-drying are typically in a range between 1% and 10% (w/v) [137]. Cyclodextrins usually yield amorphous glasses.

As discussed above, parent cyclodextrins, which are known to form intramolecular hydrogen bonds, hindering them to form hydrogen bonds with other molecules, are likely to exhibit little degree of water-replacement but should still be able to form amorphous glasses and stabilize according to the vitrification hypothesis. Proof for this can be found in a study on LDH, in which various cyclodextrins, including parent-CDs and derivatives (HP α CD, HP β CD, SBE β CD, M β CD, HP γ CD) as well as disaccharides were investigated, all in concentrations of 1% (w/w) [176]. All hydroxypropylated derivatives as well as M β CD yielded higher relative activities than the parent cyclodextrins and SBE β CD. The relative activity of LDH freeze-dried with the parent cyclodextrins was on the same level as LDH freeze-dried with trehalose and sucrose. The authors found that a concentration of 1% is too low for water replacement by disaccharides and that the increased activity observed for hydroxypropylated CD derivatives is due to their additional hydroxyl moieties, possibly contributing to hydrogen-bonding. There are numerous further reports of failure or at least of inferiority of parent cyclodextrins as compared to hydroxypropylated derivatives with regard to protein stabilization, but in turn this could also be attributed to their lack of surface activity [167,171,172,178].

In contrast, in cases in which the formation of amorphous glasses was shown to be sufficient to preserve protein stability, parent cyclodextrins can well fulfill this task, as demonstrated in a study on spray- and freeze-drying of trypsin and BSA [170]. Relative activity for spray-dried trypsin in the presence of amorphous α -CD was 95% and 92% for freeze-dried trypsin, respectively. The authors stated that the mechanism of stabilization is unclear, but glass forming excipients seemed to have a higher ability to preserve the enzyme's activity, compared to partly crystalline sucrose and mannitol.

3.4.7. Protein stabilization by cyclodextrins during storage of lyophilizates

Besides the aforementioned protein-stabilizing mechanisms during freezing and drying, there are some additional factors influencing storage stability of lyophilizates. These are for example high glass transition temperatures (T_g) of the lyophilized cake [182,183] and an optimal residual water content (usually <1% w/w) [184]. Furthermore, crystallization of initially amorphous excipients might be harmful to the dried protein [185]. Little is known about glass transition temperatures of freeze-dried cyclodextrins with only some published values available. Due to its high molecular weight, parent β -CD possesses a rather high glass transition temperature. Reported T_g -values of freeze-dried β -CD are 55 °C at a water content of 2.5% (w/w) in combination with invertase and 108 °C at a water content of 2.5%–3.5% (w/w) in combination with IL-2 [167,177].

There are some hints available in literature that storage stability of CD-containing lyophilizates can be attributed to the high glass transition temperatures of cyclodextrins. In an early study on an antibody, HP β CD at a concentration of 5% (w/v) was employed to freeze-dry an IgG monoclonal mouse antibody [174]. Recovery of the antibody immediately after lyophilization as measured by ELISA was above 80% even without any excipients and was not improved by the addition of HP β CD, sucrose or dextran. However, during subsequent storage for 18 days at 56 °C, the HP β CD-containing formulation yielded higher levels of antigen binding than the sucrose- and dextran-formulation. Residual moisture levels of the freeze-dried cakes were 1–2% (w/w), probably resulting in storage close to or above the T_g of sucrose but most likely below the T_g of HP β CD and dextran [177,186].

The high glass transition temperature of β -CD was probably also beneficial in a study on freeze-dried formulations of the cytokine IL-2. During storage at 45 °C for 36 weeks, β -CD prevented protein aggregation more effectively than sucrose and dextran but slightly less than trehalose. In addition to their high T_g -values, cyclodextrins,

remaining amorphous can inhibit crystallization of other excipients during storage of lyophilizates. This was the case with β -CD at a concentration of 10% (w/v) in combination with trehalose [167]. In another study, HP β CD was used at a concentration of 0.5% in combination with mannitol (1.5%) for freeze-drying of TNF [175]. After storage for one month at 37°C, HP β CD-containing formulations showed intermediate levels of non-reducible dimers and well preserved bioactivity. The stabilizing effect of HP β CD during storage of the lyophilizates was attributed to vitrification.

3.5. Cyclodextrins as folding aides/artificial chaperones

It is reported that CDs can be used *in vitro* as folding assistants. An example is the application of CDs as protein folding aids for carbonic anhydrase B [187]. This involves a one-step technique with the CD introduced in the solution of the denatured protein. The CD transiently interacts with the non-natively folded protein thereby supposedly shifting the equilibrium from intermolecular interaction between the peptide segments towards intramolecular association that favors natural refolding. This technique is often referred to as “dilution additive mode” [54,188,189]. Further examples involve studies that are also listed in Table 4, e.g. on aminoacylase [128] and recombinant mink and porcine growth hormone [63].

In other studies CDs have even been found to behave as artificial chaperones [51,190–193]. The endogenous GroE chaperone system served as a model for the development of an artificial chaperone system. Like the endogenous GroE chaperone system the artificial chaperone acts by a two step mechanism. In the first step binding of a detergent that captures the non-native protein prevents aggregation but on the other hand also renaturation. In a second step the detergent is stripped away from the protein by the addition of a suitable stripping agent.

Cyclodextrins have proved to be useful as stripping agents in the cases of CAB [188], citrate synthase [53] and lysozyme [54]. As these three proteins are structurally very different from each other a general applicability of the system can be assumed. Fig. 3 gives an overview on the different ways of artificial refolding of non-native proteins.

4. Application of cyclodextrins in liquid and dried protein formulations

In the following two paragraphs general recommendations for the rational use of cyclodextrins in protein formulations are given. These considerations are based on the most important mechanisms of stabilization that were discussed in the previous sections.

4.1. Liquid formulations

In summary, two main situations can be distinguished when considering the application of cyclodextrins in liquid protein formulations. Depending on the structure of the therapeutic protein either direct interaction with certain CD-derivatives in bulk solution is observed or no interaction can be detected. As discussed in detail in Section 3.2, the rather weak and transient protein–CD interaction can be analytically captured by techniques such as surface plasmon resonance spectroscopy or calorimetric methods.

If direct interaction between CDs and a therapeutic protein is observed it is likely that the addition of CDs to the protein formulation will affect stability under a variety of stress conditions, for instance thermal stability during quiescent storage as well as stability during surface-induced stress. As discussed in detail in Section 3.3.1 direct interaction between CDs and therapeutic proteins often leads to shielding of hydrophobic interaction and therefore to a reduction of protein aggregation rates, relatively independent of the trigger leading to protein unfolding. However, it has to be kept in mind that also some cases of reduced thermal protein stability were reported

in the presence of CDs. As with other excipients in protein formulation, empirical tests are required to determine the exact effects of CDs on protein stability.

If there is no direct interaction between CDs and a therapeutic protein in bulk solution the main application of CDs is usually the inhibition of surface-induced aggregation that occurs for instance during agitation or freeze-thawing processes. CDs such as HP β CD may then be used as an alternative to non-ionic surfactants. As discussed in Section 3.3.2 concentrations as low as 2.5 mM of HP β CD may be sufficient for the complete inhibition of protein aggregation. In these cases the thermal stability of the investigated protein is usually unaffected by the presence of CDs.

4.2. Dried formulations

In Section 3.4 an overview on studies investigating cyclodextrins as excipients in dried protein formulations was given. Based on the detailed overview of the studies in Section 3.4 suggestions for the rational application of CDs in dried protein formulations are provided. As a first step, it is recommended to decide which purpose the CD should serve in the formulation. The purpose will determine the choice of derivatives as well as the concentration range to be tested. If a surfactant-like effect is desired, the choice will be on surface active-derivatives at comparably low concentrations; if CDs are supposed to serve as lyoprotectants, higher concentrations of amorphous, hydrogen-bond forming derivatives will be selected.

The concentration of CD needed for adequate stabilization of proteins during freeze-drying is rather low if a surfactant-like effect is desired. From Section 3.4.5 it can be deduced that for HP β CD concentrations of approximately 0.1% (w/v) up to 1% (w/v) are most effective and a further increase in concentration does not provide further stabilization against interfacially-induced stress conditions.

CDs may also act as lyoprotectants when used at higher concentrations (larger than 1% (w/v)). In some cases the addition of CDs to protein formulations was found to be beneficial, because high glass transition temperatures can be achieved allowing for good physical stability of the lyophilized cakes at elevated temperatures.

As with CDs in liquid protein formulations another aspect should take into account the acceptance of CDs as excipients in parenteral products. If only CDs that are already used in approved parenteral products should be used, HP β CD is certainly the excipient of choice. Because of its surfactant-like properties, the ability to act as amorphous stabilizer and its toxicological safety, HP β CD meets the requirements as valuable excipient for freeze-dried formulations.

5. Conclusions

Cyclodextrins are a very promising class of excipients for the stabilization of liquid protein formulations. A wealth of studies demonstrating the capability of cyclodextrins to inhibit therapeutic protein aggregation is available. Generally, it is assumed that cyclodextrins interact with hydrophobic parts of proteins thereby suppressing protein aggregation via these exposed hydrophobic parts. Indeed many studies show good evidence for this mechanism to be in place. However, this mechanism of stabilization seems to work only for certain proteins with a structure allowing for the complexation of hydrophobic residues by CDs. Other effects of cyclodextrins on proteins, that depend less on the structure of the protein, have also been observed. For instance, cyclodextrins were shown to inhibit protein aggregation induced by exposure to the air–water interface indicating surfactant-like effects of cyclodextrins. However, the underlying mechanisms of stabilization against aggregation at the air–water interface are not fully understood yet.

Altogether it remains challenging to predict if a certain CD-derivative will be suitable for the stabilization of a liquid formulation of a particular protein. Protein conformational and colloidal stability are

the result of a complex interplay of different influencing factors and apparently the ring size of the cyclodextrin used may have as much an influence as the nature of substituents or the degree of substitution. Finally, also the presence of charged groups on CDs is of significant importance for the effects on protein aggregation. More studies on the effects of CDs on pharmaceutically relevant therapeutic proteins at high concentrations would be desirable.

Summarizing the different studies on the use of CDs in lyophilized protein formulations, it can be stated that cyclodextrins, especially the substituted derivatives, are valuable excipients. Different mechanisms of stabilization seem to be exerted simultaneously. From the fact that protein stabilization can already be achieved at low concentrations of cyclodextrins and that surface active cyclodextrins (hydroxypropylated and methylated derivatives) are most effective at inhibiting aggregation it can be deduced that surfactant-like effects of CDs seem to play an important role in the mechanism of stabilization. In addition there is evidence that sugar-like effects, most importantly water replacement, play an important role. The formation of an amorphous glass alone (vitrification), as provided by parent CDs, can be insufficient to ensure adequate protein stability. However, the high glass transition temperatures observed in protein formulations containing cyclodextrins can contribute to the physical stability of freeze-dried products, for example by preventing crystallization of amorphous stabilizers.

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