

## Protein-Protein Interactions Leading to Aggregation: Perspectives on Mechanism, Significance and Control

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(Received 4 June 2010, Accepted 2 July 2010)

Protein aggregates, whether amorphous or structured (amyloid), have attracted much attention in recent years and despite extensive efforts, the mechanism of their formation is poorly understood. While "natural" aggregation (polymerization) of monomers could improve the biological function of some proteins, it is usually the darker side of this phenomenon which is discussed in many studies: deleterious aggregation that could lead to loss of biological activity under *in vitro* conditions or cause misfolding diseases. In this review, protein aggregation has been overviewed, starting from some general concepts involved in its formation, followed by mentioning studies aimed at elucidation of its kinetics and mechanism, or characterization of intermediates that would be aggregation-prone, and finally, reporting some of the studies related to the design of methods that would control the process. Similarly, amyloid aggregates have been defined, and current methods used in their characterization have been briefly described, with an emphasis on *in silico* studies. Finally, identification and design of such molecules which may be effective in control of this process is discussed.

**Keywords:** Aggregation, Amyloid, Protein, Interaction, Chemical modification, Small molecules

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### INTRODUCTION

In recent years, "aggregation", has become a topic of high interest in the scientific community. Searching for a non-specific definition for aggregation results as: "the collecting of units or parts into a mass or whole, and the condition of being so collected" (<http://www.merriam-webster.com>).

The general idea that is derived from such a concept may appear positive, but in reality, it is a process involved in a

number of events leading to loss of biological function and occurrence of pathological conditions.

Aggregation of proteins results from the assembly of individual molecules, and is driven by the forces involved in protein-protein interactions. "Natural aggregation" occurring during polymerization of proteins may be of functional significance, with the possibility of making them more efficient, or sometimes less active. As examples, monomeric bovine isocitrate dehydrogenase is inactive, and dimerization is needed for its activation [1], while baker's yeast hexokinase is active in its monomeric form and shows lower affinity for glucose upon dimerization [2].

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Deleterious aggregation could hamper the production and storage of a therapeutically or industrially important protein (*in vitro*), which could end into the production of inactivated species, and even toxic intermediates, or be a concern related to the safety of the product that could become immunogenic [3]. Protein aggregates could also take various forms in the so-called “misfolding” diseases. These unnatural “ensembles” are usually separated into “amorphous” aggregation, and “amyloid” structures.

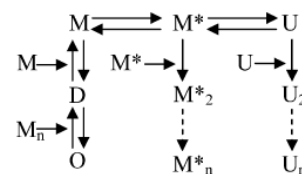
In this review, various facets of protein aggregation have been discussed, from the basic physicochemical rules controlling the process, to the more practical issues related to its prevention. As examples, studies have been presented, including our own, dealing with characterization of the process using methods such as chemical modification and environment engineering, including employment of immobilization strategies and addition of various additives. Theoretical methods such as molecular dynamics simulation have also been included.

## GENERAL CONCEPTS

A scheme has been proposed which takes into account the various forms of a protein, with an emphasis toward amorphous aggregation [4]. The native protein (M) could reversibly go toward intermediate species (M\*), and aggregate in a higher order process to M\*n species [5] (Scheme 1). Among the various intermediate forms, the so-called molten globule state is generally considered to be frequently involved in this process. This intermediate structure may be obtained by removing protein-bound metal ions or cofactors in the presence of moderate concentrations of denaturants [6].

There is still a possibility for the intermediate structures to fully unfold (U) and form aggregates. In this scheme, the “self-association” of proteins (*i.e.* their natural polymerization) is also taken into account, since (D) represents the dimer form, and O is an oligomeric form, both species being reversibly formed.

It has been postulated that the same forces that may conduct a protein toward folding, could lead to its aggregation. Exposure of hydrophobic groups to the solvent may then result into a kinetic competition between folding and aggregation [6]. This concept bears in itself the importance of intermediate



Scheme 1 [4]

and partially unfolded states of the protein.

A successfully folded protein structure is the result of a fine balance between hydrophobic and Coulombic forces, usually stated as being “marginally stable” [7]. Limited proteolysis experiments, leading to the production of individual fragments of various lengths that could be subsequently reassembled, has been the basis of the suggestion that elements of secondary structures would be the bearer of this marginal stability and proceed to the contacts that would ultimately end in tertiary structure formation [8].

Hydrophobic interactions are believed to be of particular importance as stabilizers of the folded state [9]. Measures of “protein stability” have been made with the use of protein denaturation/renaturation and values as low as 5-15 kcal mol<sup>-1</sup> have been obtained for the conformational stability of globular proteins [10]. This  $\Delta G_D$  is representative of the difference between free energies of the folded and unfolded states; thus changes in these energies that could be the result of changes in intramolecular interactions of these two states, could affect stability of a protein [11]. Comparative studies involving the use of mesophilic and thermophilic enzymes, has led to the conclusion that subtle structural differences between these proteins suffice to influence stability [12,13].

Every little change could then affect the unique and functional folded form, with direct results on its ability to interact with other protein molecules. Changes in environment (pH, salt concentration, solvent, temperature, pressure), or in the protein structure (amino acid composition, chemical modification), even those which may sometimes be very subtle, could have dramatic manifestations [14-16].

Given the importance of this phenomenon, various methods have been devised to follow formation of protein aggregates, detect them, try to extract kinetic models from such information, and even predict their formation. Of the most widely used detection methods are spectroscopic ones:

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UV-Vis absorbance/turbidity, intrinsic or extrinsic fluorescence spectroscopy, as well as light scattering. On the other hand, more sophisticated methods that allow better detailed characterization of the process such as NMR, EM (electron microscopy), and AFM (atomic force microscopy) are now gaining more attention [17]. The use of multiple techniques has been suggested to avoid over-interpretation and to ensure that accurate kinetic data are collected [17-19]. Different existing kinetic models of aggregation have been dissected in a recent review [17], where various “approaches”, categorized as kinetics/thermodynamics, empirical, and “others”, include mass action equations, QSAR equations, non-mass action equations, logistic equations and prion aggregation mechanisms.

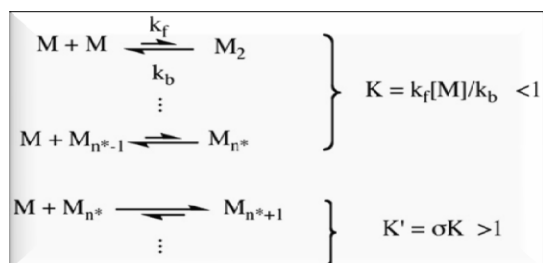
As a matter of fact, the kinetics and mechanism of protein aggregation have been of interest for approximately fifty years [17]. In spite of extensive reports on the subject, however, the mechanism of protein aggregation is poorly understood, and while there seems to exist physical evidence for the starting structures and final products of protein aggregation, further information is still needed for a better characterization of such “toxic” intermediate species [20,21].

The initial phase of protein aggregation involves the monomeric structure of a protein, which is able to be converted to an active form by many factors from which some are discussed in the following sections of this review. In these active forms, exposed “sticky” hydrophobic patches provide the protein with a higher propensity to aggregate. There is no consensus about protein aggregate formation *via* a mechanism involving nucleation [22,23].

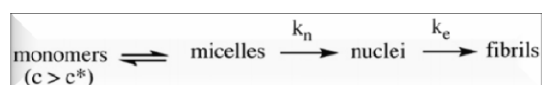
In a recent review Morris *et al.* have organized five classes of kinetic mechanisms [17] that will be mentioned briefly here.

### The Subsequent Monomer-Addition Mechanism

As suggested by Kasai *et al.*, the aggregation of native state G-actin to F-actin involve positive cooperation interactions [24]. Later this mechanism was applied to kinetics of sickle-cell hemoglobin gelation [25], where distinction was also made between the nucleation and polymerization steps. In Scheme 2, this mechanism is represented with M standing for monomer,  $k_f$  and  $k_b$  being the forward and backward rate constants, respectively, and  $n^*$  denoting the critical nucleus size. The constant that describes the addition steps during



Scheme 2. [25]



Scheme 3. [28]

nucleation is assumed to be less than one, but becomes more than one during polymerization.  $K$  indicates a constant with a favorable reverse reaction, while  $K' = \sigma K$  indicates a favorable forward reaction. A “critical nucleus” is considered to represent species which are of the lowest thermodynamic stability in solution. These are minimally sized oligomers capable of initiating further growth.

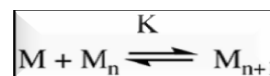
Variations of this mechanism include homogenous and heterogeneous nucleation on the surface of existing polymer [26], and a model for the self-assembly of microtubules from tubulin [27] involving aggregate formation during the nucleation phase, and irreversible steps of monomer addition in the next stages.

In 1996, fibrillation of high concentrations of beta-amyloid ( $A\beta$ ) (0.1 mM) was modeled within this mechanism by Lomakin *et al.* Here, aggregation is slowed down by acidic conditions, and could be followed (over time) by quasi-elastic light scattering (QLS) [28]. This proposed model is presented in Scheme 3, where  $c$  represents protein concentration,  $c^*$  the critical micelle concentration (CMC),  $k_n$  the rate constant for nucleation, and  $k_e$  the rate constant for elongation. At protein concentrations above CMC ( $c > c^*$ ), (i) “protein micelles” are formed, (ii) nucleation occurs within these structures, and (iii) elongation takes place on the nuclei (by irreversible binding of monomers to the fibrils’ ends). For protein concentrations

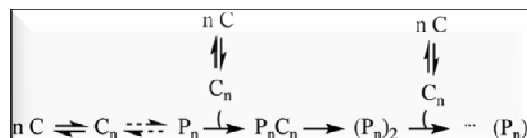
below CMC ( $c < c^*$ ), no nucleation occurs so that (seeded) growth may only take place on impurities [28]. Other researchers have since proposed that soluble oligomers of A $\beta$  are protein micelles [29].

### The Reversible Association Mechanism

This class of mechanism can be simply shown in Scheme 4 [30], for which an example is the reversible association of glutamate dehydrogenase. Here a variation of the reversible association, termed the random association mechanism, is proposed in which two units (monomeric or polymeric) of any size can associate to form a larger polymer [31].



Scheme 4. [30]



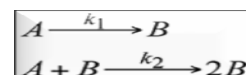
Scheme 5. [32,33]

### Prion Aggregation Mechanism

When studying aggregation of yeast prion proteins, Linquist proposed the Nucleated Conformational Conversion mechanism (NCC) as shown in Scheme 5 [32,33]. Here formation of C nuclei is followed by slow conversion to P nuclei. Once P nuclei are present, further assembly is thought to occur rapidly. Formation of larger aggregates involves the P nuclei acting as templates, combining with C nuclei [32]. In this mechanism, the infectious species is a P nucleus ( $P_n$ ), and propagation occurs through assembly and conversion of C nuclei ( $C_n$ ).

### An ‘‘Ockham’s Razor’’/Minimalistic 2-Step Model

This class of kinetic mechanisms was first proposed in 1997 by Watzky *et al.*, describing transition-metal nanocluster formation [34], and was recently shown to apply to a broad spectrum of aggregating proteins, including  $\alpha$ -synuclein, A $\beta$ , polyglutamine and prions, related to Parkinson's, Alzheimer's, Huntington's and prion diseases [35,36]. In Scheme 6 provided below, A represents a ‘‘precatalytic’’ form of the protein monomer while B represents any ‘‘catalytic’’ aggregated form of the protein past the critical nucleus size. Hence, all aggregates able to perform autocatalysis are treated as kinetically equivalent species in this minimalistic kinetic model. Importantly, the rate constants  $k_1$  and  $k_2$  correspond to nucleation and growth, respectively, so that this two-step model specifically and easily separates (average) nucleation from (average) growth. Also, as species B is both a catalyst and a product in the growth step reaction, the second step ( $A + B \rightarrow 2B$ ) is considered to define autocatalysis.



Scheme 6. The Finke-Watzky (F-W) mechanism [35] that has been applied to 41 aggregation data sets [35,36]

### Quantitative Structure-Activity Relationship Models

A phenomenological approach to protein aggregation that uses physicochemical properties of a protein was originally developed by Chiti *et al.* [37]. Here, these investigators looked at the effect of amino acid mutations on protein aggregation rates. Their approach is to correlate the observed aggregation rate changes to calculated changes in protein physicochemical properties such as hydrophobicity, charge, and propensity to convert from  $\alpha$ -helical to a  $\beta$ -sheet secondary structure [37]. It was shown that the hydrophobicity and hydrophobic/hydrophilic patterns (or the propensity to convert from  $\alpha$ -helix to  $\beta$ -sheet) contributed positively to the aggregation rate, while charge contributed negatively.

This leads us to another relatively recent development in the field, which is the advent of predictive tools which aim at finding correlations between protein sequence (and parts of structures) and the ability to aggregate.

The rationale behind these efforts is that not all regions of a polypeptide would be equally important for the aggregation tendency both in natively unfolded and globular proteins. Among the more widely used tools developed in this regard are Aggrescan, Tango, Zyggregator, Pasta, and Betascan. The

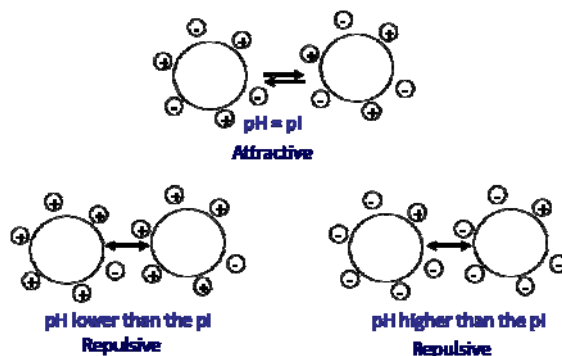
basis underlying some of these program algorithms (such as Zygggregator and Aggrescan) is the aggregation propensity of each residue as a combination of different factors including charge, hydrophobicity, tendency for secondary structure formation and pattern of hydrophobic and hydrophilic interactions [38-40]. These algorithms are generally based on experimental data derived from inducing variations in residues of specific proteins and peptides [37]. In the Tango program calculation of conformational energy is done as a phase-space of major class of secondary structure for buried residues. On the other hand, in Pasta and Betascan, sequence-specific interaction energies between favored residues-pairs of protein fragments are considered, and the programs often predict segments for parallel beta sheet formation [41,42]. These programs lack predictive capacity for oligomer formation and other aggregates that are devoid of beta structural features. Also, they are not able to fully take environmental factors into account. It should however be mentioned that in some cases, high correlation has been found between predicted and actual experimental results [43,44].

From a practical point of view, factors affecting the intensity of aggregation are of particular importance. It is then logical that in a considerable number of studies related to protein stability and conformation, one part is devoted to investigate ways to inhibit or diminish the rate of aggregate formation. In the following sections, some representative studies carried out by the authors of this review have been summarized with the aim of offering a concise practical overview of some of the above-mentioned concepts.

## ASSOCIATION OF PROTEIN MOLECULES: AMORPHOUS AGGREGATION

A first step in studying the phenomenon of protein aggregation is identification of the factors that could increase protein aggregation.

If aggregation is viewed as a manifestation of protein-protein interactions (PPI), then forces that are of importance in these interactions should be studied. There has been a concern as to whether known components of PPI in native proteins would be the same in structurally altered proteins, but in cases where aggregating species have minor differences with the native state, this is not a problem [45]. Forces involved in PPI



**Fig. 1.** Importance of net charge in the aggregation propensity of protein molecules.

include specific and non-specific interactions: hydrogen bonding, van der Waals interactions and steric interactions that have been suggested to play minor roles in dilute solutions of proteins in their native states. Additionally, electrostatic and hydrophobic interactions are considered to play major roles [46,47]. In cases where high concentrations of proteins exist in solutions, however, the relative contribution of these forces would be different [48].

When considering protein molecules as polyelectrolytes, a consequence of having equal positive and negative charges on these molecules ( $\text{pH} = \text{pI}$ ) would provide a higher possibility of attraction between them, while imbalance of these charges would result in repulsive forces, distancing the molecules from each other (Fig. 1). Aggregation is thus increased when changes occur in the protein structure that decrease net charge: mutation or chemical modification of charged amino acids are among examples of such changes [14,49-54]. As a matter of fact, electrostatic factors affect protein viscosity, and when high net charge exist on the protein molecule, electroviscous effect has a significant contribution to its flow behavior in solution [4], thereby influencing the extent of interactions.

As an example of the effect of modifying charges on protein solubility and aggregation, a study involving chemical modification of lysine residues of a mesophilic alpha-amylase could be mentioned. Use of citraconic anhydride resulted in conversion of positive charges of these residues to negative ones at neutral pH. A somewhat unexpected observation was that addition of calcium, a normal stabilizer for the enzyme, was deleterious to the modified form and resulted in pronounced aggregation upon heating. The effect was thought

to be directly linked to thermo-inactivation of the modified form, and was non-specific since other divalent ions were also effective. Accordingly, charge neutralization was proposed to be the main cause of thermo-inactivation occurring *via* aggregation [51]. In the same line of experiments, neutralization of lysine residues of lysozyme, which occurred by acetylation, was suggested to be at least one of the factors causing increased aggregation [54]. It was also demonstrated that mutations which change protein net charge toward neutrality, may favor aggregation [55].

### Intermediate Structures

**Molten globular forms, protein hydrophobicity.** A critical event triggering aggregation of a protein is partial unfolding, and since molten globular forms are considered to be major intermediate structure species, any factor causing destabilization of a protein toward molten globular structure formation would be expected to increase its aggregation. Molten globular forms are, by definition, compact partially folded species possessing secondary structure similar to native forms, but lacking the specific tertiary native structures [56-59]. These forms have been suggested to be similar with some states which appear during early refolding events of several proteins [56,60-63].

Higher exposure of hydrophobic sites is considered to be a factor driving protein structures toward molten globular states [64]. This kind of exposure was demonstrated to be dramatically enhanced for cytochrome c at pH equal or lower than 4.0 [65]. Under such conditions, the protein was found to acquire the potential for interaction with a hydrophobic matrix [65]. This observation was later developed into provision of a method for adsorptive immobilization of proteins [66-71] and subcellular structures [72-75] *via* hydrophobic interactions. Reversible denaturation involving exposure of hydrophobic sites in protein molecules provided a new tool for adsorptive immobilization by hydrophobic interactions [76-78].

**Apo forms.** Another way of generating a higher propensity to aggregation would be enhancing hydrophobic exposure of the structure as it occurs in the transformation from the holo-form of a metallo protein to its apo- counterpart. Separation of the structurally essential metal ion from a holo-enzyme could be performed by the relatively simple method of incubating the enzyme with a chelator and subsequent dialysis or use of a

chromatographic technique [79]. It has been indeed observed that upon removal of zinc from yeast alcohol dehydrogenase and bovine carbonic anhydrase by the use of 1,10-phenanthroline, the structures gain higher flexibility and show exposure of hydrophobic patches, as monitored by circular dichroism spectra and fluorimetric methods (*e.g.* ANS). The subsequent decrease in thermostability and increase of aggregation intensity have been then monitored by the use of UV-Vis spectroscopy [77,80]. A recent study on thermal stability of human carbonic anhydrase has also shown lower thermal stability, which was related to a higher thermal mobility on the surface of the enzyme and not in the vicinity of the active site [81]. Specific structural features could also be of importance, as for the apo form of a camel alpha-lactalbumin [82]. Higher stability has been observed compared to the bovine counterpart (both being more unstable than the holo form), and the discrepancy has been attributed to different extents of hydrophobic interactions [82]. It should be mentioned that in some cases, (for example sterol carrier protein-2) the apo-enzyme would be able to maintain structural stability [83]. In case of a multiple-binding metalloenzyme metallothionein, the apo-form has been suggested to have indeed a physiological role, and has been observed to be non-specific related to the type of metal ion it can chelate [84].

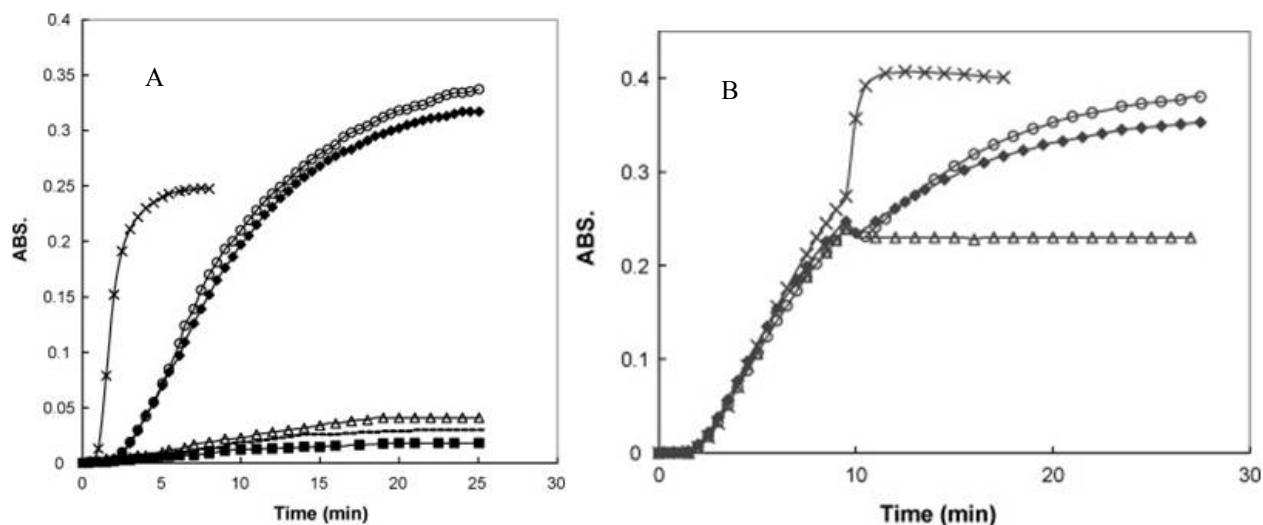
## PREVENTING AGGREGATION

Starting from the fact that the main event is the occurrence of protein-protein interactions, any method that is able to diminish these contacts should be effective in prevention of aggregation. Protein structure stabilization *via* changes in protein microenvironment or structure manipulation, divert the protein-protein interactions by supplying an alternative scaffold (as surfaces), and especially, use of a wide array of small molecules, acting at various stages of the aggregation process, are examples of these approaches.

### Effect of Ligands

An interesting class of small molecules that may dramatically affect aggregation, are allosteric regulators of enzymes, which alter protein conformation.

During a study involving thermal denaturation of GDH



**Fig. 2.** A. Aggregation of GDH in the presence of effectors. Enzyme alone (◆), enzyme with 50 mM leucine (■), enzyme with 0.1 mM NAD<sup>+</sup> (-), enzyme with 0.1 mM ADP (▲), enzyme with 0.5 mM GTP (○), enzyme with 0.1 mM NADH (×). B. Effect of addition of effectors in the middle of the aggregation process: 0.5 mM GTP (○), 0.1 mM NADH (×) and 0.1 mM ADP (▲). Reprinted from [85], with permission from Elsevier.

effective induction and protection of aggregate formation was observed by the use of some “specific ligands” interacting with this well-characterized allosteric protein. Positive effectors (leucine, NAD<sup>+</sup>, ADP) were found to diminish, and even almost completely prevent aggregation while the negative effector NADH increased the rate of aggregate formation with a shorter lag phase. An interesting feature was the fact that these ligands were able to affect the aggregation process even when added in the middle of its progress (Fig. 2).

Circular dichroism spectra showed also a perfectly preserved structure of the enzyme when incubated with ADP. Flexibility of the enzyme is thought to be of importance in this case, with positive effectors such as ADP rigidifying the protein structure, and negative effectors, including GTP, acting in an opposite manner. These fine effects are conveyed throughout the protein structure *via* subtle local conformational changes that are brought by the presence of specific ligands; in other words, it is the environment of the allosteric protein that is regulating its function [85]. Allostery may be an intrinsic property of all proteins [86], and structural flexibility is important for all protein functions [87].

## Polyols

Polyols have been successfully employed to indirectly prevent aggregation, that is, as refolding aids. Low concentrations of polyols, including sugars, have been successfully used to increase the refolding yield of proteins [88-90]. Classical studies have also shown the general potential of polyols in stabilizing protein structures, which has been related to an entropic effect with the reasoning that the free energy change of transfer of a denatured protein from water to aqueous media (containing polyols) would be larger than that of the native protein. Protein stabilization would be achieved as a result of a solvent ordering effect [91]. On the other hand, another study has suggested that for linear polyols, increase in hydroxymethyl chain length could possibly be related to a more effective stabilization of hydrophobic interactions [92].

Recent studies on thermodynamics parameters have also shown that while polyols could increase the T<sub>m</sub> of model proteins in direct relationship with their concentrations, they manifest an effect on the Gibbs energy of stabilization of proteins at lower pHs (with no effect at neutral pH) against

thermal denaturation [93,94].

Individually, polyols have also been studied with regard to their specific effect on proteins, sometimes with various effects. As an example, erythritol was found to increase thermal stability of proteins [95], while failing to be effective in refolding of aggregation-prone citrate synthase [96]. Other examples include the stabilization effect of glycerol related to chemical and thermal denaturation conditions because of its solvophobic effect [97,98], ethylene glycol having a mild effect of destabilizing protein conformation [91], and sorbitol increasing the  $T_m$  of some proteins [91,92]. A linear relationship has also been established between the partial molar volume of these polyols and their effect on protein stabilization [95], with an extension to a relationship between the number of hydroxyl groups and an effect on thermal stabilization for example on a highly thermolabile protein [99], and also their effect on refolding of aggregation-prone protein [96] or stabilization of a protein molten-globular form [100]. As another practical example, apo- form of yeast alcohol dehydrogenase, that was described in the previous section to have more hydrophobic sites exposed to solvent was shown to be protected against thermal denaturation and aggregation with the use of mannitol, sorbitol, sucrose, and trehalose, with sucrose giving the best result. An interesting point was the observation that the apo-enzyme incubated in the presence of sucrose or trehalose was even more thermally stable than the holo-enzyme in the absence of the protecting agent. These polyols were suggested to play a stabilizing role resembling the effect of the structural metal. A similar result has been observed also for the apo- form of carbonic anhydrase [77]. A general stabilizing effect of sugars has been proposed to be due to an increase in the cohesive force of the medium and lowering of protein diffusion [80].

### Chaperone-Like Substances

The threat of aggregation appears from the very first moments of protein existence in the cell. This problem worsens with changes that happen in their environment, such as an increase in temperature. One of the survival strategies that cells employ in such cases is an increase in chaperone synthesis [101]. These molecules have been shown to be able to prevent protein aggregation, and disassemble preformed aggregates [102].

Some compounds, such as casein, with “chaperone-like” properties have been found to be general inhibitors of protein aggregation, affecting the initial partial unfolding stage of the process [103]. Variation in aggregation-preventing effect has been observed, depending on the polarity of N-terminal region of casein [104], or more generally, on its net charge, and surface hydrophobicity which was demonstrated for camel and bovine caseins [105,106]. In one study involving yeast hexokinase B, the presence of casein and its subsequent removal by cyclodextrin was effective in protecting the enzyme from aggregation and denaturation, presumably by a “chaperone-assisted refolding” mechanism [107].

For GDH, the allosteric protein whose aggregation was specifically affected by the presence of effectors, the partial aggregation prohibiting effect of casein and cyclodextrin was suggested to be related to their ability to interact with intermediates bearing a higher hydrophobic surface, again approving a certain “general” property for these ligands [108].

For another model protein, namely an inhibited form of alpha-chymotrypsin, thermal aggregation has been shown to be dependent on hydrophobic factors, as seen from the hydrophobic exposure over the surface of the enzyme (assessed by fluorospectrometry). The opposite effects of kosmotropic and chaotropic anions, and the beneficial effect of chaperone-like compounds such as alpha-crystallin (which binds to hydrophobic patches of the unfolded enzyme molecules) have been observed [109]. Existence of patches of positive charges was suggested to be of importance in this process, since chemical modification of lysine residues was shown to reverse lack of protein aggregation observed at acidic pH [109].

### Polyamines and Amino Acids

Stabilization of protein structures is directly related to the preventive potential of some amino acids (such as proline and arginine) on aggregation. Arginine has been used successfully as a refolding aid, while its effect on aggregation suppression has been suggested to occur on partially unfolded proteins and structure intermediates [110-113].

In a study investigating a more precise mechanism, arginine has been compared to GdnHCl, with which it shares the ability of interacting favorably with many proteins’ side chains, but differs with regard to its binding on protein

surfaces, which is relatively more limited [114].

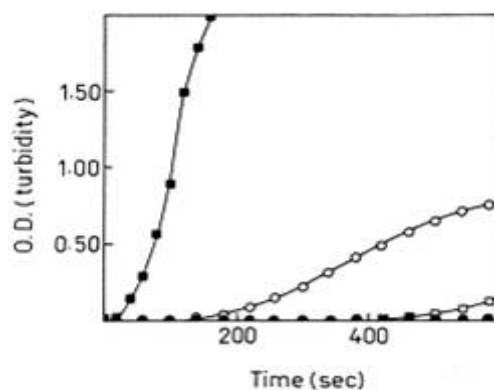
Interestingly, another group of compounds has gained more attention as aggregation preventers in the recent years. These compounds show some structural similarity with the side chain of arginine and are collectively referred to as “polyamines”. Polyamines include spermine, spermidine, putrescine, and agmatine, are linear structures of varying lengths, bearing amine groups, and have been shown to be derived from ornithine or arginine [115]. These compounds have been shown to be essential for cell survival [115], and capable of protecting proteins against thermal inactivation [116,117]. A study on the thermoprotective effect of polyamines on lysozyme has led to the suggestion that a possible explanation for this effect was the formation of ion pairs with local negative charges on the protein, with a resulting increase of the net charge and increased electrostatic repulsion [112].

In a study on protein aggregation involving alpha-chymotrypsin, intermediate concentrations of trifluoroethanol (TFE) were found effective in promoting protein-protein interactions. As demonstrated by the effect of temperature on this process, hydrophobic factors were found to be important, with the aromatic residues more solvent accessible in the conformation induced by TFE. Electrostatic interactions were also found to play a significant role as demonstrated by experiments involving changes in salt concentration, pH variation and modification of lysine residues [118]. In this case, polyamines (such as putrescine, spermidine, spermine), were found to inhibit both thermal and TFE-induced aggregation of alpha-chymotrypsin, possibly *via* kosmotropic effects [53].

A “general” aggregation suppressive property has been suggested for these compounds [112], but they may also interact in a more specific manner with proteins, as proposed in the case of GDH [108].

### Chemical Modification

Counteracting the aggregation propensity of proteins with more drastic methods has been achieved *via* chemical modification of their surface residues. This method has been shown to provide a tool for improving protein stability. Chemical modification of specific residues could also be considered as an imitation of post translational modification,



**Fig. 3.** Aggregation of native and modified forms of BAA at 70 °C. Native BAA in the absence (○) and presence (□) of additional calcium; 12 residue-modified BAA in the absence (●) and presence (▪) of additional Ca<sup>2+</sup>. Reprinted from [51], with permission from Elsevier.

which may confer novel properties to synthesized proteins, as, for example, higher stability. Accordingly, modification of lysine residues was found to dramatically enhance thermal stability of a mesophilic alpha-amylase (*Bacillus amyloliquefaciens* alpha-amylase), at high temperatures. Resistance of modified mesophilic enzyme toward irreversible thermoinactivation was found to be accompanied by diminished aggregation and deamidation of the protein, two important mechanisms of irreversible thermoinactivation (Fig. 3). Reversal of charge, and providing new opportunities for hydrogen bonding with the modified group attached to lysine residues could be suggested to have a role in this regard [51]. Modification could also diminish surface hydrophobicity without affecting net charge, and lower the aggregation propensity of the protein [119].

In the same line of studies, the effect of homobifunctional cross-linkers providing intramolecular cross links between lysine residues was also investigated on thermal stabilization of this enzyme as also observed for a number of other proteins [120,121]. A modified form obtained using a succinimide ester was found to gain higher stability due to a probable rigidification of the structure [52].

In a study on the effect of glycosylation on protein aggregation, glucoamylase was used as a model protein. This enzyme was treated *in vitro* with mannosidase in order to

obtain a deglycosylated form, whose properties were then compared with the native (glycosylated) enzyme. It was found that this modified enzyme possessed lower thermal stability, concordant with a higher degree of aggregation. Higher surface hydrophobicity resulting from the removal of carbohydrates was proposed as one important factor in this regard, as a probable role for exposure of aggregation-prone beta-sheets which are normally shielded by carbohydrates. As an additional observation, there was a lack of any detectable aggregation at alkaline pH which was suggested to be related to repulsive interactions that occurred due to a significant difference between the pH at which the experiment was carried out and the pI of the protein (3.5-3.7) [122], again highlighting the complexity of this phenomenon and the need to consider the various factors that could be affecting protein-protein interactions.

## AMYLOID FORMATION

### General Concepts

**Introduction.** Aggregate structures are usually classified as amorphous (considered to be more disordered), and amyloid, or amyloid-like which are highly ordered and contain cross-beta sheets [37,55,123]. Presence of cross-beta segments has also been reported in amorphous aggregates [37,55,123], but these are flexible, and could not be compared with the quasi-crystalline structure of amyloid fibers [124].

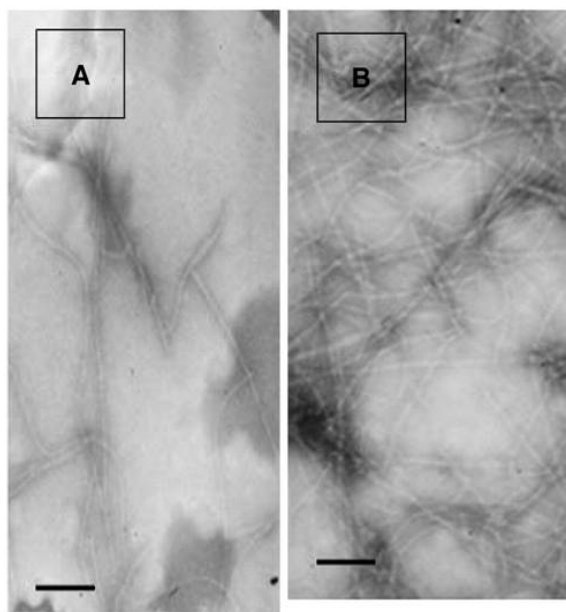
As a matter of fact, using slightly different environmental conditions would lead to a different state of aggregation, as shown about alpha-chymotrypsin as a model. In this case, alpha-chymotrypsin was driven toward amyloid-like fibril formation, with an absence of amorphous aggregates, when the pH was acidic and either high temperature or low TFE concentration was applied. Conversely at neutral pH, heating the protein or incubating it with TFE at intermediate concentrations yielded large amorphous aggregates. In this study, it was suggested that the amyloid fibrillar state of a protein could be reached by a variety of structurally distinct conformational ensembles, since the aggregation process could start from conformations with various secondary and tertiary structural characteristics, but a necessary condition would be that the solution condition allows formation of intermolecular interactions (especially of aromatic and pi-

stacking nature) and inhibits the alternative pathway of amorphous aggregation [125]. Some studies suggest that more native like conformations with high percentage of buried aggregation-susceptible residues would preferably form amorphous structures (*vs.* amyloid) [126], while another point of view states that formation of amorphous aggregation need harsher condition [127].

Many studies involving amyloid structure formation aim toward description of the events which may occur during the transformation of the starting structures, and are often accompanied by discussions as to how they could be controlled. Formation of amyloid structures could include intermediate, amyloid-like, to classical beta-crossed fibrils and even native-like structures which could be partially unfolded [128]. The concept of native-like aggregation is suggested to be more general than previously believed, and involves native-like states that undergo limited local perturbations. These events could be for example accessed transiently *via* structural fluctuations facilitated by mutations, or more stably *via* more severe mutations. These native-like forms are proposed to be responsible for aggregation under physiological conditions [129].

As in the case of amorphous aggregation, impact of various interactions (hydrophobic, electrostatic,...) related to specific types of residues has been experimentally studied on this process with the use of mutant model proteins [37] or chemical modification of specific residues. As an example, modification of lysine residues in lysozyme with acetic anhydride (acetylation) resulted in the formation of species which would be driven more easily toward fibrillation (Fig. 4). On the other hand, use of citraconic anhydride (citraconylation) led to the formation of a modified enzyme that would be less prone to form amyloid structures [54]. Similarly, a chemical modification study on insulin showed a precedently unrecognized role for the single lysine residue of this protein. In this case too, citraconylation of the lysine residue resulted in a dramatic decrease of amyloid formation under acidic conditions [130].

**Modeling studies.** It should be pointed out that detailed atomic-level information of the fibril structure doesn't exist as yet [131], nor do we have a good understanding of the processes involved in fibril self-assembly [132]. In these conditions, many studies use *in silico* simulation method in



**Fig. 4.** Overview of HEWL fibrils by TEM. Transmission electron microscopy images of non-modified (A) and chemically acetylated HEWL (B). Samples were incubated at pH 2.5 and 57 °C for 32 h. Scale bars represent 240 nm. Reprinted from [54], with permission from Elsevier.

order to gain a better insight into the intriguing phenomenon of various proteins acquiring beta-sheet rich structures.

Since there exists a wealth of experimental data on abeta peptides, many simulations have targeted their structural changes or the design of potential inhibitors of their fibrillation. Studies on abeta peptides encompass a wide range of various structures, from fragments [133-135], to full-length monomers and oligomers [136-139]. With the use of molecular dynamics simulation methods (and especially replica exchange), precise suggestions could be made as to the variation of hydrogen bonds in various pH conditions, and the structural parts and interactions that would be of importance in each condition [135,136].

Other studies involve model proteins such as insulin [140], lysozyme [141], and disease-related proteins including transthyretin, microglobulin, synuclein, and the prion protein [142-145]. A number of studies take a more general approach, in order to derive conclusions as to the cause of the existing

variations in fibril morphologies [146], or on the contrary, characterization of common segments (for example Q/N rich regions) that would be of general importance in fibril formation [147].

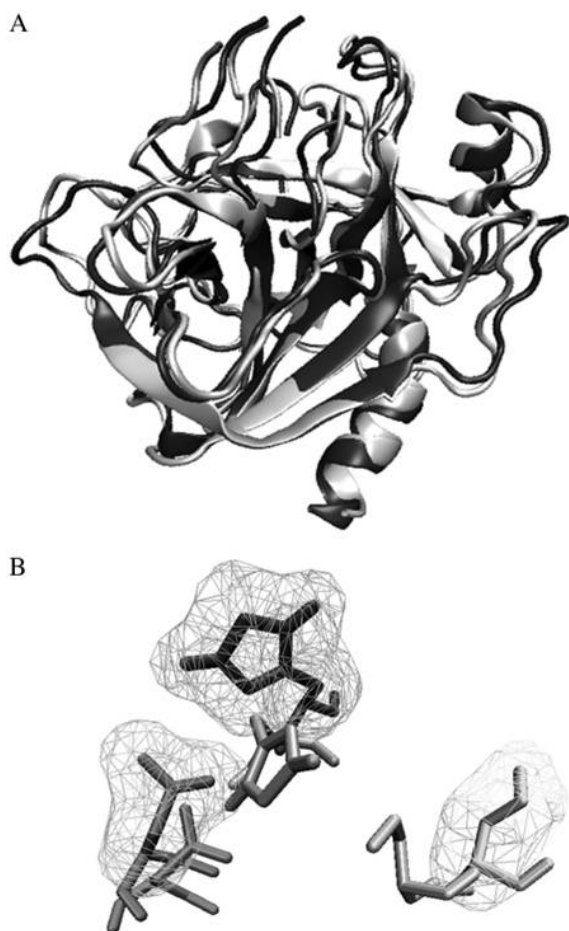
An alternative way of probing regions of importance in aggregation is the study of *in silico* mutation effects on amyloidogenic abeta peptides [134,139,148].

A different approach involves use of non aqueous solvents as medium during simulations. Examples include methanol, in which the importance of solvophobic effects has been studied in the self-assembly of an A $\beta$  fragment [149], and ethanol, which has been used alongside with high temperature to study amyloid formation in lysozyme [141]. Other solvents include trifluoroethanol (TFE). In the case of humanin, a stabilizing role has been found using TFE instead of with water [150], while for alpha-chymotrypsin this solvent was shown to distort the catalytic site (Fig. 5) and result in an increase of the hydrophobic solvent-accessible surface areas of the enzyme [151].

As a last example of these studies, simulations should be mentioned in which membrane models have been used for abeta [152] and synuclein [153] in order to determine their possible deleterious effects on biological membranes.

### Preventing Amyloid Structure Formation

Preventing "amyloid" formation is the subject of many studies, involving many efforts toward the discovery of new therapeutics for the ever-growing number of known misfolding diseases. The balance between native protein, misfolded protein, oligomers and fibril structures may be influenced by a variety of factors. Overproduction of an amyloidogenic protein, its unusual covalent modification or cleavage, failure to normal degradation, and insufficient molecular chaperone activities may all contribute to driving an otherwise normal system toward a disease state. Each of these steps may be considered as a potential target for an effective therapeutic intervention [128]. It should be mentioned that intermediate structures formed during partial unfolding of proteins which are on their way to form amyloid structures have been shown to possess more cytotoxicity in comparison with the final fibrils [154,155], and as such, are currently the center of attention [156]. Characterization of these structures involves use of various methods [157]. These are capable of



**Fig. 5.** (A) Ribbon representation of the average structures of  $\alpha$ -chymotrypsin in water (light gray) and TFE/water mixture (dark gray) after fitting. (B) Average structure of the catalytic triad (Ser<sup>195</sup>, His<sup>57</sup>, and Asp<sup>102</sup>) of  $\alpha$ -chymotrypsin in water (bare) and TFE/water mixture (covered by wire frames). Reprinted from [151], with permission from Elsevier.

measuring folding events on microsecond timescale using ultra-rapid mixing [158], or detecting rare species (*e.g.* using FRET, fluorescence correlation spectroscopy (FCS) or NMR spectroscopy) [159,160].

Small molecules that could effectively interfere to these stages have gained much attention during the recent years, and cover a vast array of different structures.

An interesting finding in the field of designing effective

small molecules to target protein-protein interactions was the realization that there exists small regions in a protein molecule which could contribute disproportionately (with regard to their size), to the energy of binding [161]. These ‘hot spots’ of protein structures have been identified by alanine scanning [162]. Thus, the idea that small molecules could be interfering between large surfaces of proteins approaching each other has regained strength [161] and is now widely seen as the background of the many studies aiming at discovering anti-amyloidogenic compounds.

These compounds range from structures resembling Congo Red and thioflavin T (probes for monitoring amyloid formation) [163,164], to known drugs used for other purposes such as rifampicin and its derivatives [165], amphotericin B [166], doxorubicin and its derivatives [167-169].

Below, a selection of small molecules used as amyloid inhibitors is presented, as well as a brief part on the issue of rational design of such compounds.

## SELECTED SMALL MOLECULES INHIBITORS OF FIBRIL FORMATION

### Well-Known Drugs

The advantage of using known drugs in studies directed to the discovery of novel anti-amyloidogenic agents is that in case of effect, there already exists a wealth of data on various properties of the drugs, such as their pharmacokinetic profile, and in most cases, derivatives of the drug have been synthesized and even at times characterized.

As an example, rifampicin was shown to inhibit A $\beta$  fibril formation and protect cells from its toxicity under *in vitro* conditions. In the same study, dapsone was shown to have no significant effect on A $\beta$  fibril formation. The idea of testing these drugs was raised from a report on leprosy patients that lacked brain senile plaques in comparison with age-matched controls, suggesting a possible effect of leprosy fighting drugs on A $\beta$ . Other compounds sharing the naphthoquinone ring of rifampicin (rifamycin SV, rifamycin B and rifamycin S) were also effective in this regard, which suggested a possible free radical scavenging role for these rings, alongside with hydrophobic interactions of rifampicin ansa chain with the peptide, blocking associations between peptide molecules [165].

Amphotericin B, which is an anti-fungal drug with other activities including inhibition of generation of the scarpie isoform of the protein prion [170], and the delaying of its accumulation in the brain [171], has also been shown to delay fibrillogenesis by targeting an amyloidogenic stretch of A $\beta$ , the 25-35 peptide [166]. Taking the toxicity of amphotericin B into consideration, the design of its derivatives has been proposed [166]. It is interesting to note that two other macrolides, mycostatin, and fillipin III are also effective inhibitors of A $\beta$ <sub>1-40</sub> but when used at higher concentrations [172].

Doxorubicin is an anthracycline used in cancer chemotherapy. Both this drug and a similar compound, 4'-iodo-4'-deoxydoxorubicin, which possess an iodine atom in place of a hydroxyl group in doxorubicin were studied and it was shown that 4'-iodo-4'-deoxydoxorubicin was able to disaggregate five types of amyloid fibrils [167]. It has also been shown that 4'-iodo-4'-deoxydoxorubicin may disrupt the fibrillar structure of transthyretin to amorphous material [168].

In a subsequent study, the interaction of this compound with a variant of transthyretin was investigated in more detail, using crystallography and molecular modelling techniques. The protein used was a L55P mutant, which was suggested to be in an "amyloid-like" state, resembling an intermediate of the amyloid formation process. 4'-iodo-4'-deoxydoxorubicin disrupted the mutant variant crystals but not the wild-type protein, suggesting that the contact region existing between the monomers of the mutant which is absent in the wild type protein is presumably the binding site of the compound [169]. Based on chemical analogy with doxorubicin, the antibiotic tetracycline, which has a safer toxicological profile, alongside with its derivative doxycycline, were tested on A $\beta$ <sub>1-42</sub>. Both compounds were able to inhibit A $\beta$ <sub>1-42</sub> fibril formation, and attenuate resistance of amyloid fibrils to proteolysis, a useful capacity for more effective clearance of amyloid fibrils [173].

Since minocycline, another tetracycline, has been shown to have neuroprotective effects in Alzheimer's disease models under *in vitro* and *in vivo* conditions [174], this chemical structure could be considered as potential candidate in anti-amyloid drug design research. Due to possible effectiveness of anti-inflammatory agents in providing protection against Alzheimer's disease [175], the effect of non-steroidal anti-inflammatory drugs (NSAIDs) was investigated for control of

fibril formation of A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub>. The drugs included ibuprofen, aspirin, meclofenamic acid, diclofenac, ketoprofen and naproxen. All these compounds were able to inhibit fibril formation in both A $\beta$ s and destabilize pre-formed fibrils, with ibuprofen and meclofenamic acid being the most effective. These findings suggest NSAIDs to be a promising candidate for combating Alzheimer's disease, through several mechanisms including suppression of chronic neuroinflammation, reducing the amount of soluble A $\beta$ <sub>1-42</sub> fibrils, and directly inhibiting the deposition of A $\beta$  fibrils in the brain [176]. Anti-Parkinsonism drugs, divided into dopamine agonists, monoamine oxidase B (MAOB) inhibitors, and anti-muscarinic agents have been assessed for their effect on A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> [177] as well as  $\alpha$ -synuclein [178] fibrillation. It was found that dopamine, L-dopa, dopamine agonists pergolide and bromocriptine and MAO-B inhibitor selegiline dose dependently inhibited fibril formation from A $\beta$  and  $\alpha$ -synuclein and destabilized preformed fibrils, while the anti-muscarinic agent trihexyphenidyl failed to be effective in this regard [177,178]. The results suggest the possibility of using common drugs for both Alzheimer's and Parkinson's diseases. The mechanism of action of these drugs has been proposed to be related to their antioxidant properties [178]. For dopamine, the possibility of forming a covalent adduct with  $\alpha$ -synuclein has also been put forward in a previous study [179], followed by subsequent report on formation of dopaminochrome, an oxidized product of dopamine [180]. However, it should be noted that while dopamine inhibits fibril formation of  $\alpha$ -synuclein, it causes an accumulation of the protofibrillar forms of the protein [179], which is thought to be more toxic than the fibrils. There is indeed a recent report on the *in vivo* toxicity of unregulated cytosolic dopamine, demonstrating that chronic exposure to this compound causes neurodegeneration. However, this toxicity has not been found to involve  $\alpha$ -synuclein, since elevated expression or accumulation of the protein was not observed [181].

### Polyphenols and Other Antioxidants

Polyphenolic compounds have attracted much attention, with their neuroprotective effect being attributed to their antioxidant properties [182,183]. However, hydrophobic interactions between phenolic rings and specific hydrophobic features of amyloidogenic proteins could also play a role

[184]. Common structural properties including hydrophobic and hydrogen bonding elements have even been proposed for carbazole and tetracyclic compounds, suggesting the existence of similar 3D conformations for different polyphenols when interacting with amyloid structures (monomer or oligomers) [185]. The inhibitory activity of some stilbenes on A $\beta$  fibril formation has been assessed with resveratrol and its monoglucosidic form (piceid) showing better activity in this regard. Among these series, smaller structures appear to be better candidates [186]. This does not appear to be a general rule, since larger flexible structures capable of making hydrophobic and numerous hydrogen bonds (tannic acid) have also been reported as being able to destabilize A $\beta$  fibrils. Tannic acid is a polymeric polyphenol which has been shown to be more potent than the flavonoid myricetin, with a much smaller structure [187]. Another series of studied polyphenolic compounds include nordihydroguaiaretic acid, curcumin, rosmarinic acid and ferulic acid [188,189]. All these small molecules have been classified as antioxidants, with curcumin, rosmarinic acid and ferulic acid having additional anti-inflammatory properties [190,191]. They are able to dose-dependently inhibit formation of fibrils from A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub>, as well as their extension. Moreover, they may destabilize preformed fibrils, which are then no more able to act as seeds [188]. As for curcumin, the effective component of curcuma spice, a possible mechanism of action could be its potential as beta-sheet breaker. These compounds have a remarkable effect on inhibiting amyloid structure formation, and a possible general effect on the amyloidogenic process of very different proteins. The anti-amyloidogenic effect of curcumin has been previously shown on the A $\beta$  peptide, alpha-synuclein, prion protein, and transthyretin [189,192-194]. In an experiment on another model protein, namely, insulin, various concentrations (ranging from 2 to 8 micromolars) of curcumin were utilised in physiological conditions (pH 7.5, and 37 °C) and shown to be effective in decreasing fibrillogenesis [130]. A structurally related compound to curcumin is salvianolic acid which has been shown to inhibit fibril formation, and possess neuroprotective properties when tested under *in vitro* conditions involving human neuroblastoma cells. It has a lesser effect than curcumin but is more active than ferulic acid. All three compounds are derivatives of caffeic acid [195]. It is interesting to note that other derivatives of curcumin

(demethoxycurcumin, and bis demethoxycurcumin) have also been shown to have potent inhibitory effects on A $\beta$  fibril formation, in contrast to tetrahydrocurcumin which is ineffective in this regard. The effective curcuminoids were found to be more potent compared with flavonoids [196], and certain synthetically-prepared derivatives of curcumin have been shown to possess  $\gamma$ -secretase inhibitory activity [197]. Such “multitargeted” compounds (for example, piperidine derivatives with dual inhibitory potency against acetylcholinesterase and A $\beta$ <sub>1-42</sub> aggregation [198], are currently becoming the object of much interest. Other antioxidants including alpha-lipoic acid [199], its metabolic product dihydrolipoic acid (DHLA) [199], coenzyme Q10 [200] and nicotine [201-203] have been studied related to A $\beta$  amyloid formation. Nicotine has attracted particular attention based on reports indicating that an inverse relationship may exist between smoking and Alzheimer's disease [204]. The neuroprotective effect of nicotine against A $\beta$  toxicity *in vitro* [205,206] and control of A $\beta$  fibril formation [201-203] have subsequently been tested. Flavonoids are polyphenolic compounds widely distributed in plants, several types of which have been shown to possess various interesting therapeutic functions including antioxidative and anti-inflammatory properties [207]. They have been found effective in controlling fibrillation of A $\beta$ <sub>1-42</sub> [196], A $\beta$ <sub>1-40</sub> [172,208], tau protein [172], and  $\alpha$ -synuclein [209]. Those which inhibited fibrillation of tau were also found effective related to A $\beta$ , suggesting a common mechanism of action [209]. Baicalein, a flavonoid from a Chinese herb was found to have the capacity of controlling  $\alpha$ -synuclein fibrillation and disaggregation of pre-formed fibrils suggesting that it acts on or prior to the nucleation phase [209]. Binding of baicalein is thought to occur in the vicinity of one or more of the four tyrosine residues of  $\alpha$ -synuclein, with another possible interaction with the central hydrophobic part of the protein (residues 61-92). Disaggregation occurs from both ends of the fibril and internal regions, possibly due to intercalation of baicalein in the beta-sheets. Overall, it has been proposed that baicalein acts *via* more than one mechanism, possibly including covalent binding with lysine residues [209]. Interestingly, some synthesized benzofuranone, which are structurally comparable to flavonoids, have been shown to influence amyloid formation in insulin, with various degrees

of effectiveness (submitted manuscript).

### Other Compounds

**Surfactants.** Amphiphilic surfactants 1,2-dihexanoyl-sn-glycero-3-phosphocholine (di-C6-PC) and 1,2-diheptanoyl-sn-glycero-3-phosphocholine (di-C7-PC) have been reported to inhibit A $\beta$  fibril formation under physiological conditions, in a concentration and time dependent manner [210].

**Indole derivatives.** Based on the facts that short peptides containing a phenylalanine pair bind to A $\beta$  fibrils, that heteroaromatic interactions in general play important roles, and that two indole derivatives (melatonin and indole-propionic acid) were reported to inhibit A $\beta$  fibril formation, a study was performed on a total of 29 indole derivatives, with the use of both A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> [211]. Three compounds namely indole-3-carbinol, 3-hydroxyindole and 4-hydroxyindole, were shown to be the most effective inhibitors of A $\beta$  fibril formation, with relatively lower effectiveness toward A $\beta$ <sub>1-40</sub>. Indole alone was not an effective inhibitor, and the presence of a hydroxyl group in a specific position was required. Indole-3-carbinol and 3-hydroxyindole were postulated to interfere in the polymerization stage of fibrillation, while 4-hydroxyindole seemed to be able to act on nucleation in addition to interfering with the polymerization step. In cell toxicity assays, it was shown that 4-hydroxyindole protected the cells from the cytotoxicity of A $\beta$  fibrils, while 3-hydroxyindole could not improve cell survival, presumably since the presence of this compound results in generation of toxic aggregates from A $\beta$ . Hydroxyindoles possess attractive properties as potential drug candidates, being small, hydrophobic, and probably able to cross the blood-brain-barrier [211]. The effect of a series of indole derivatives were also tested on amyloid fibril formation from hen egg white lysozyme. Although similar compounds have been reported to act as inhibitors of this enzyme [212], and a docking experiment showed the active site ready to accommodate these compounds, they did not protect the HEWL native state from conformational change. As monitored by ThT fluorescence, CD spectra and TEM images, they diminished HEWL amyloid fibril formation, delaying both the nucleation and elongation phases. An interesting effect was also the disaggregation of previously formed HEWL amyloid fibrils (enhanced by indole 3-acetic acid). Overall, a preliminary structure-activity

relationship could be devised as the indole ring possessing the main role in effectiveness of ligands, a positive role for the side chain hydroxyl group and a negative role for the side chain carbonyl and intervening methylene groups [213].

### Small Stress Molecules

Small organic molecules produced under stress conditions (*e.g.* thermal stress) in microorganisms or plants, including ectoine, betaine, trehalose and citrulline were presumed to be effective against amyloid fibril formation based on their capacity to enhance protein stability and activity under harsh conditions [214]. Subsequent observations indicated the capacity of ectoine, trehalose, and citrulline to cause complete inhibition of insulin fibrillation [214]. Ectoine and hydroxyectoine have been tested on A $\beta$ <sub>1-42</sub>, where both compounds had concentration-dependent inhibitory effect on fibril formation. The greater effectiveness of ectoine has been attributed to its higher hydrophobicity, possibly leading to better interaction with A $\beta$  in the positions where hydrophobic interactions may cause association of the protein molecules into aggregate formation. The actual needed concentration of these compounds as potential drugs was estimated to be lower than 1 micromolar. Other advantages of these compounds are their high compatibility with cell metabolism, the tolerance of their producer organisms toward them (up to 1 M concentrations), and the probability of crossing the blood-brain barrier. In the same study, the effect of ectoines was shown on prevention of A $\beta$ <sub>1-42</sub>-induced cell toxicity, with ectoine being more effective [214]. On the other hand, a recent study aimed at elucidating mechanism of amyloid inhibition by trehalose involving two model proteins indicated while delay/inhibition of fibrillation are obvious, more toxic oligomeric species may be formed during the process [215]. This again highlighted the need for more detailed studies on small molecules that inhibit amyloid fibril formation. Mannosylglycerate, a natural extremolyte that could be addressed as a small stress molecule, has also been found to be effective in inhibiting A $\beta$ <sub>1-42</sub> fibrillation and in prolonging the lag time for the formation of amyloid oligomers. It is interesting to note the relative lack of efficacy of its structural analog  $\alpha$ -D-mannosylglyceramide, and structurally related mannose, methylmannoside, and glycerol. This has led to the suggestion that a carboxyl group in mannosylglycerate could

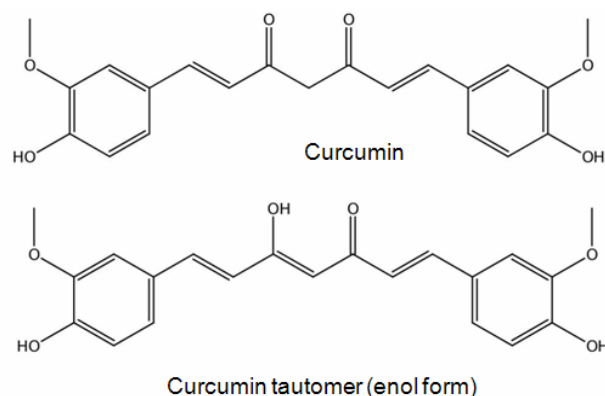
be the important factor in its effect. Mannosylglycerate has also been shown to increase cell survival rate in an assay with human neuroblastoma cells exposed to amyloid toxicity [216]. Finally, another study has been performed on the inhibitory effect of ectoine and mannosylglyceramide on the amyloid fibril formation of prion peptide 106-126, showing their ability for partial inhibition of aggregation and neurotoxicity of this peptide. Interestingly, mannosylglycerate had no effect in this case, with a possible explanation being that the carboxyl group interacts only with the polar head of the peptide, while its hydrophobic tail is involved in formation of amyloid aggregates [217].

### Other Natural Compounds

Natural products have attracted much attention as a rich source in the drug discovery process in the recent years. Among these, indigenous plants are subject of more interest. In one study, two components of saffron have been tested on amyloid formation of apo-lactalbumin. These compounds were previously reported to be effective against growth of malignant cells [218], and beneficial in cases of learning impairment [219]. Both compounds were shown to inhibit fibrillation of this protein, with crocin inhibiting oligomer formation, and possibly acting as a beta-sheet breaker [220].

### HOW A SEARCH FOR FINDING EFFECTIVE SMALL MOLECULES MAY BE RATIONALIZED?

In medicinal chemistry approaches, where the ultimate goal is to design an effective drug, a lead compound is first identified, which is a structure possessing properties that could be therapeutically useful. The lead compound is usually the starting point, from which other analogous structures are synthesized to determine how structural variations affect pharmacological activity. This is known as structure-activity relationship (SAR). Besides this ligand-based approach, in cases where a target structure is known, drug design strategies could be initiated from the binding site of the structure, in order to find compounds with good fit. In the quest for potential anti-amyloidogenic drugs targeting fibril formation of proteins, both methods have been used to some extent, but approaches describing rational design strategies have been



Scheme 7. Curcumin keto and enol structures

limited to only a few cases. A logical starting point is to define SARs for each known group of effective small molecules that affect fibrillation. Such attempts have been made for curcumin-like compounds [221], and tetracyclines and their analogues [185,222] as well as for carbazole derivatives [185]. In the case of curcumin-like compounds, the first step was identification of a core in curcumin structure (Scheme 7). Based on analogy with Congo Red and chrysin G, that share the ability of binding to fibrils, a chemical scaffold composed of two aromatic rings separated by a rigid, planar backbone has been proposed. Features of this scaffold were investigated in a library of compounds and the following results were obtained: two aromatic groups are probably needed for optimal interactions, effective ligands bear some substitutions on aromatic rings, and flexibility and length of the two aromatic groups linker may affect activity of the ligands [222].

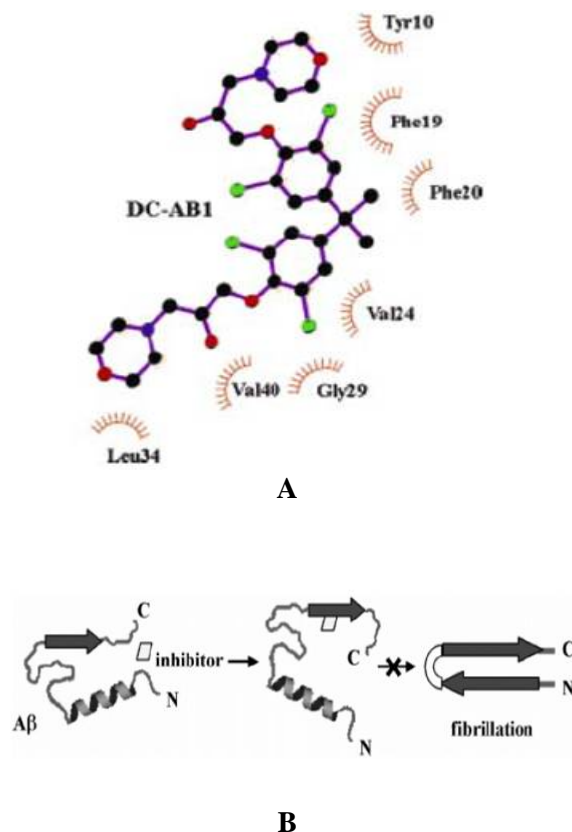
The fibril formation of  $A\beta_{1-42}$  in the presence of these compounds was monitored, and for comparative purposes, an  $IC_{50}$  was obtained as the half-maximal concentration of the compounds required for inhibition. It was suggested that the binding site of curcumin and its analogues must contain hydrophobic and hydrogen-bonding groups, that two phenyl groups were essential for activity, that the distance between the two interacting aromatic groups should be between 8 and 16 Å, and that a hydroxyl substitution on the aromatic end group was necessary for inhibition. An interesting result was that flexibility and length were not independent variables.

## Protein-Protein Interactions Leading to Aggregation

Resveratrol provided an exception in that while it possessed a short linker, it proved effective, having probably a different binding site [221]. In a separate study where isoxazole and pyrazole derivatives of curcumin were prepared, some SAR interpretations were made [197]. A search for common effective groups and their relative positions in space (pharmacophore) has been made on rolitetracycline and daunomycin as well as the carbazole carvedilol. These three compounds were shown to be effective in inhibiting  $A\beta_{1-40}$  amyloid fibril formation, and could be superimposed to access a common 3D conformation [185]. Four common features were found, namely, two aromatic centers, one donor atom and one acceptor site. Inactive compounds could possess the required motifs but did not have the conformational flexibility to access the required conformation. The pharmacophore was also found in carvedilol metabolites, which are also active compounds [185].

Another study has been made, again with the aim of identifying a pharmacophore in tetracycline and its analogues, using molecular mechanics and *ab initio* methods, but the results were not conclusive for finding a geometrical pharmacophore differentiating between active and inactive compounds. Therefore, properties other than geometrical ones were postulated to be crucial in determining anti-fibrillogenic activities such as stereo-electronic properties of the compounds [222]. An interesting study in this regard was made with targeting a computationally prepared model of an  $A\beta_{1-40}$  intermediate structure [223]. Since  $A\beta_{1-40}$  adopts a variety of conformations in aqueous solutions [224], it is difficult to choose a structure as a target for a new inhibitor design. A molecular dynamics simulation made with  $A\beta_{1-40}$  in order to simulate its conformational transition to amyloid structure, has revealed that the peptide undergoes an alpha-helix/beta-sheet intermediate structure during the process [225]. The structure had a core domain (residues 24-37) of which four glycine residues were essential for beta-sheet formation [225]. This intermediate structure was then chosen as target with the aim of finding compounds capable of binding to the beta-sheet region of the intermediate that would interrupt formation of pleated beta-sheet of amyloid structures. Thus docking of molecules was made computationally into this binding site, and from the 125 compounds that were bound favorably within the site, one was experimentally found

to be able to inhibit fibrillation of  $A\beta_{1-42}$ . Putative interactions of this molecule (named DC-AB1) with a hydrophobic site of the C-terminal region in the modelled structure of  $A\beta_{1-40}$  are shown in Fig. 6a [223].



**Fig. 6.** A. Interaction details of DC-AB1 with  $A\beta_{1-40}$  according to the complex model. DC-AB1 binds to the C-terminal  $\beta$ -sheet region mainly through hydrophobic interactions. There are three major hydrophobic sites at the  $A\beta_{1-40}$  peptide surface that hold the hydrophobic chemical groups of DC-AB1. Two phenyl rings of DC-AB1 interact with the hydrophobic sites consisting of residues Val24, Gly29, and Val40 or Tyr10, Phe19, and Phe20, respectively. The morpholine group of DC-AB1 interacts with the hydrophobic residues of Leu34 and Val40. Reprinted with permission from [223]. B. Schematic representation of a mechanism for DC-AB1 inhibition of  $A\beta$  fibrillation. Reprinted with permission from [223].

A summary of the suggested mechanism of action of the ligand is shown in Fig. 6b [223].

## CONCLUSIONS

The field of protein aggregation enjoys a long history of efforts made toward elucidation of the mechanisms related to its formation and control. In spite of this, many details required for a clear definition of the nature of the initial participating structures and those formed at later stages in the course of the events involved in its formation is still lacking, suggesting the complexity of the related phenomena. More complex are the processes leading to amyloid structure formation which is a biological hallmark of several late-onset neurodegenerative disorders, including Alzheimer's Parkinson's and Huntington's diseases. Here, an attempt has been made to overview the field of protein aggregation, including some of the experiences of the authors of the review. It starts with description of the mechanisms involved in formation of amorphous and fibrillar aggregates, including nature of the intermediate structures, and the type of interactions involved in the provision of various "unnatural" ensembles, with possible reasons for loss of biological activity and cytotoxicity. The biological significance of such outcomes and a brief description of the various strategies taken to control some of the events leading to aggregate formation is described. Special emphasis is made in the later parts of the discussion regarding use of small molecules for the control of the various steps leading to formation of amorphous and fibrillar protein assemblies.

## ACKNOWLEDGMENTS

This work was supported by grants from the research council of the University of Tehran and the Iran National Science Foundation (INSF).

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