Protein aggregation: folding aggregates, inclusion bodies and amyloid

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Aggregation results in the formation of inclusion bodies, amyloid fibrils and folding aggregates. Substantial data support the hypothesis that partially folded intermediates are key precursors to aggregates, that aggregation involves specific intermolecular interactions and that most aggregates involve β sheets.

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Introduction

Protein aggregation can be merely a nuisance factor in many *in vitro* studies of proteins or it can cause major economic and technical problems in the biotechnology and pharmaceutical industries. Its effects can be lethal in patients who suffer from a variety of diseases involving protein aggregation, such as the amyloidoses, prion diseases and other protein deposition disorders [1,2]. This review focuses on the basic mechanism(s) of protein aggregation, the factors that determine whether it will occur, and the conformation of the protein molecules in the aggregate. Protein aggregation is intimately tied to protein folding and stability, and also, in the cell, to molecular chaperones. The prevalence of protein aggregation is probably much higher than generally realized — it is often ignored or worked around, and in protein folding experiments its presence may not even be realized [3]. The growing recognition of the critical importance of protein aggregation has resulted in a number of reviews [4–10].

Unless specifically noted to the contrary, in this review the term aggregation will apply to aggregated protein involving the formation of insoluble precipitates that may be considered 'pathological' in nature. This is in contrast to the insolubility of the native state due to protein concentrations exceeding the solubility limit (e.g. 'salting out'), or the intermolecular association involved in the formation of native oligomers. It should be noted that in many such cases of pathological aggregation the initial material formed may be soluble aggregates, but these become insoluble when they exceed a certain size.

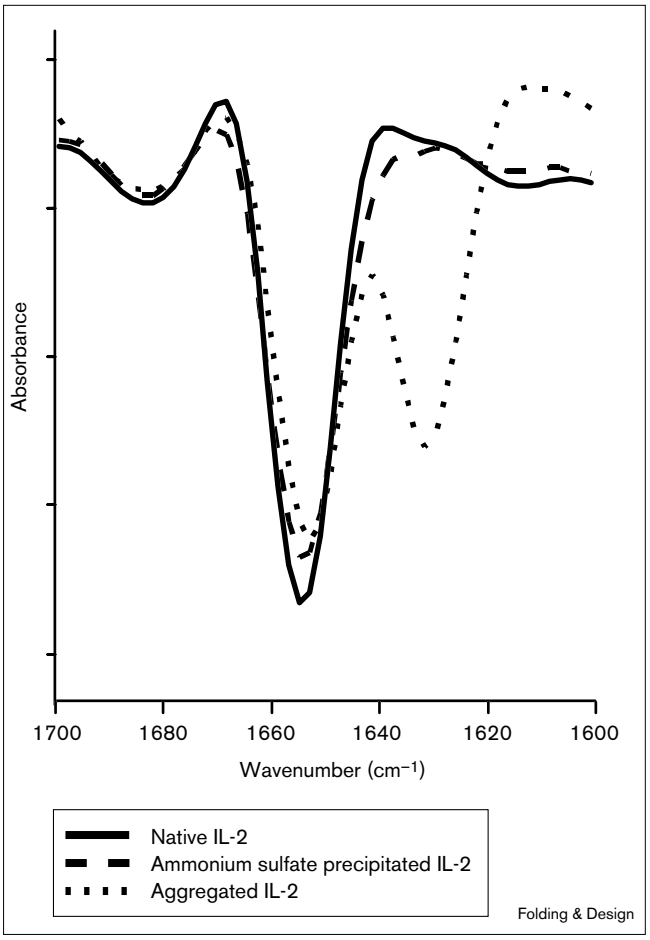
It is convenient to classify protein aggregation according to the following categories: *in vivo* and *in vitro*, and ordered and disordered. Amyloid fibrils (both *in vivo* and *in vitro*) are examples of ordered aggregates, whereas inclusion bodies are examples of *in vivo* disordered aggregates. Corresponding disordered *in vitro* aggregates are those formed during the refolding of denaturant-unfolded protein at high protein concentrations, or under weakly native conditions at high protein concentration; these will be referred to as folding aggregates.

Native, folded proteins may aggregate under certain conditions, most notably salting out and isoelectric precipitation (when the net charge on the protein is zero). Such precipitates of native protein are readily distinguished from pathological aggregates by their solubility in buffer under native-like conditions. In contrast, pathological aggregates dissociate and dissolve only in the presence of high concentrations of denaturant or detergent. In my laboratory, it has been shown that the native conformation is retained in salting out precipitates (Figure 1).

Protein aggregation has usually been assumed to involve either unfolded or native states. Inclusion body formation and other aggregates formed during protein folding have been assumed to arise from hydrophobic aggregation of the unfolded or denatured states, whereas amyloid fibrils and other extracellular aggregates have been assumed to arise from native-like conformations in a process analogous to the polymerization of hemoglobin S [8]. Recent observations suggest that aggregation is much more likely to arise from specific partially folded intermediates, however. An important consequence of this is that aggregation will be favored by factors and conditions that favor population of these intermediates, and hence it is the properties of these intermediates that are important in determining whether aggregation occurs. Furthermore, the characteristics and properties of the intermediates may be significantly different from those of the native (and unfolded) conformation.

Several observations indicate that transient aggregation occurring during *in vitro* protein refolding may be mistaken for a transient intermediate [3]. Direct evidence for the transient association of partially folded intermediates during refolding has been obtained in small-angle X-ray scattering experiments of apomyoglobin [11,12], carbonic anhydrase and phosphoglycerate kinase [13]. The experiments show the rapid (milliseconds or less) formation of associated states that become monomeric on a slow timescale (typically seconds to minutes or longer). Another approach indicative of transient aggregation is that involving changes in the rate constants for refolding as a function

Figure 1



Precipitates of native protein, in this case interleukin-2 (IL-2) formed by 'salting out' with ammonium sulfate, retain the native conformation. The figure shows the second derivatives of the FTIR spectra of the amide I region of native, ammonium sulfate precipitated IL-2 (dashed line) and, for comparison, aggregated (inclusion body) IL-2 (dotted line). IL-2 is an all-α protein, as indicated by the dominant band at 1654 cm^{-1} for the native conformation.

of protein concentration [3]. This study indicates that transient aggregates can easily be mistaken for structured monomers and could be a general problem in time-resolved folding studies. Because aggregation is sensitive to protein concentration, monitoring the kinetics as a function of concentration should reveal potential aggregation artifacts.

Key questions relating to aggregation, many of which are not yet fully answered, include the following: the nature of the species responsible for aggregation: the detailed mechanism that leads to aggregation and the underlying kinetics scheme; the structure of the aggregates; the specificity of the intermolecular interaction (e.g. are the aggregates homogenous?); why aggregation (even of the same protein) sometimes leads to ordered aggregates (amyloid) and sometimes to disordered aggregates (inclusion bodies, folding aggregates and amorphous deposits); how the environmental conditions affect the rate and the amount of aggregation; and how the aggregation may be prevented.

It seems likely from an evolutionary perspective that proteins have evolved to avoid sequences that result in a strong propensity to aggregate. It is also interesting to consider that many short peptide sequences containing several hydrophobic residues and a high tendency for β-sheet formation probably have a strong disposition to form aggregates and/or amyloid fibrils. It is only the flanking sequences, which are either quite polar and therefore increase the solubility limit or are sufficiently bulky to sterically prevent the required interactions, that result in the lack of aggregation and/or amyloid formation.

Problems due to protein aggregation

Protein deposition diseases

Several dozen protein deposition diseases are known. The most familiar include the amyloid diseases (amyloidoses), such as Alzheimer's disease, and the transmissible spongiform encephalopathies (TSEs; prion diseases such as bovine spongiform encephalopathy, BSE, or Mad Cow disease and Creutzfeldt–Jacob disease, CJD, in humans). In both amyloid and prion diseases the aggregated protein is usually in the form of ordered fibrils. Amyloid fibril formation has been observed to arise from both peptides and proteins. Several protein deposition diseases involve non-ordered protein deposits; some examples are inclusion body myositis, light-chain deposition disease and cataracts. Many thousands of people die each year from protein deposition diseases [14]. New diseases are added to this list every year, one of the latest being Huntington's disease [15].

Inclusion bodies

Inclusion body formation is very common when proteins are overexpressed. This may facilitate their potential purification because inclusion bodies are usually highly homogeneous. The problem is that renaturation is frequently difficult, as a result of aggregation. Several techniques have been developed to help overcome the common problem of their re-aggregation during renaturation [16] and some are discussed later. Inclusion bodies and related insoluble non-ordered protein aggregates are also found in certain diseases.

Protein drugs

Protein aggregation is also a problem in a number of other aspects of biotechnology; for example, during storage or delivery of protein drugs. There are several reports that protein aggregation can occur during lyophilization of proteins or during their subsequent rehydration, depending on the conditions (e.g. the water content of the system is critical [17–22]). Because in some cases it appears that the dehydration of proteins, which occurs in lyophilization,

results in denaturation [23,24], it is probably the ensuing rehydration that leads to aggregation, due to the formation of partially folded intermediates during the refolding (see below).

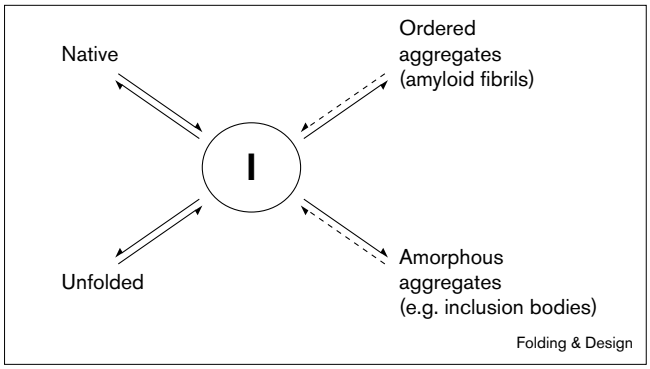
Mechanisms of aggregation

One of the earliest and most prescient studies of protein aggregation was that of Goldberg and coworkers [25] on the enzyme tryptophanase, which revealed an intermediate at moderate denaturant concentration that aggregated. Evidence for the potential specificity of aggregation was also observed in that the addition of other folded proteins did not affect the amount of aggregated tryptophanase. More recently, the idea that partially folded intermediates might be responsible for aggregation has been championed by King and coworkers [4,26,27] and Wetzel [6,8,10,28]. Recent reports supporting the involvement of partially folded intermediates in the aggregation of several proteins suggests the generality of the phenomenon [29–31].

Hypothesis

Substantial data support the following model for the formation and structure of protein aggregates, in which specific intermolecular interactions between hydrophobic surfaces of structural subunits in partially folded intermediates are responsible for the aggregation. Key features of the model (Figure 2) are as follows. Protein folding involves intermediates, each consisting of an ensemble of closely related substates (the various substates of a given intermediate will be characterized by having common secondary structure and most likely a common core of relatively native-like structure, with the remainder of the polypeptide chain disordered or in unstable structural units). The native state is formed by the sequential interaction of substructural units, the building blocks (typically subdomains), which may be stable or metastable on their own, but are stabilized by the interactions with other such building blocks [32]. Formation of the native state involves the intramolecular interaction of the hydrophobic faces of structural subunits (Figure 3a). Specificity will arise from a variety of features, of which the geometric shape and extent of the hydrophobic patches, the constraints of the polypeptide chain, and the presence of other structural subunits are probably the most important. Aggregation occurs when these hydrophobic surfaces interact in an intermolecular manner (Figure 3b). Thus, the initial stages of aggregation are quite specific in the sense that they involve the interaction of specific surface elements of the structural subunits of one molecule with 'matching' hydrophobic surface areas of structural subunits of a neighboring molecule. Three-dimensional propagation of this process leads to large aggregates. Initially, the aggregates (e.g. dimers and tetramers) will be soluble, but eventually their size will exceed the solubility limit. The fact that they may still have significant solvent-exposed hydrophobic surfaces would also minimize their solubility. The intermediates are more prone to aggregate than the unfolded state because in the unfolded state the hydrophobic sidechains are scattered relatively randomly in many small hydrophobic regions, whereas in the partially folded intermediates there will be large patches of contiguous surface hydrophobicity, which will have a much stronger propensity for aggregation (these are the surfaces that 'normally' interact in an intramolecular manner to form the native conformation). The role of domain (or subdomain) swapping [33] in aggregation is unclear, but it is certainly possible that in some cases it may be an important factor.

Figure 2

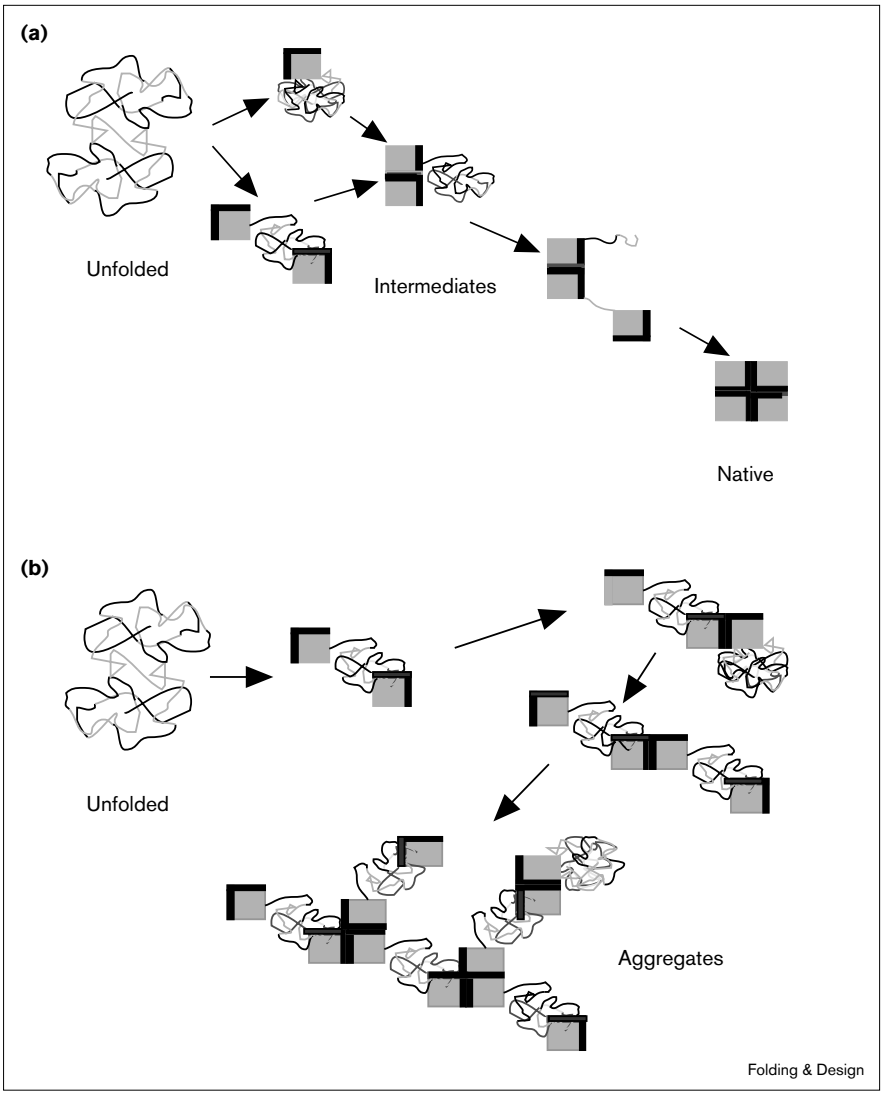


The basic model for protein aggregation. The circled I represents a key partially folded intermediate, which can be populated either in the folding direction (from unfolded) or from the native state. The intermediate I has a strong propensity to aggregate, leading to either ordered or disordered (amorphous) aggregates. It is possible that there is very local order even in apparently (macroscopically) amorphous aggregates. Intermediate I will normally be an intermediate on both the *in vivo* and *in vitro* folding pathways.

Aggregation often appears to be irreversible, but this is usually a reflection of the very slow rates of disaggregation and the fact that the equilibrium lies far in favor of the aggregate rather than its soluble monomeric form. Under certain conditions, aggregates, including *in vivo* amyloid deposits, can be reversed [34,35]. In practice, however, once insoluble aggregates form, the process is effectively irreversible under native-like conditions.

A number of observations suggest that specific interactions are involved in protein aggregation. Among the clearest evidence for such specificity is the work of Brems and coworkers [36–40] with bovine growth hormone (bGH), which showed that a peptide fragment of bGH could inhibit aggregation and that mutations, which increased the hydrophobicity of the domain interface, increased the propensity for aggregation. Another strong indication of specificity in aggregation is the homogeneity of inclusion bodies [41], which are normally highly homogeneous once any contaminating material has

Figure 3



Proposed mechanisms for aggregation involving structural building blocks (subdomains). See text for more details. **(a)** The normal folding situation, in which hydrophobic surfaces (black) of the structural units interact in an intramolecular manner to form the native conformation. Each identifiable intermediate along the pathway will consist of an ensemble of substates, having in common the compact structural unit(s), with the remainder of the chain in a disordered or unstable structured form. **(b)** The situation in which aggregation occurs by intermolecular interaction of the structural building blocks, again via interaction of complementary hydrophobic surfaces. The common intermediate in folding and aggregation can be seen.

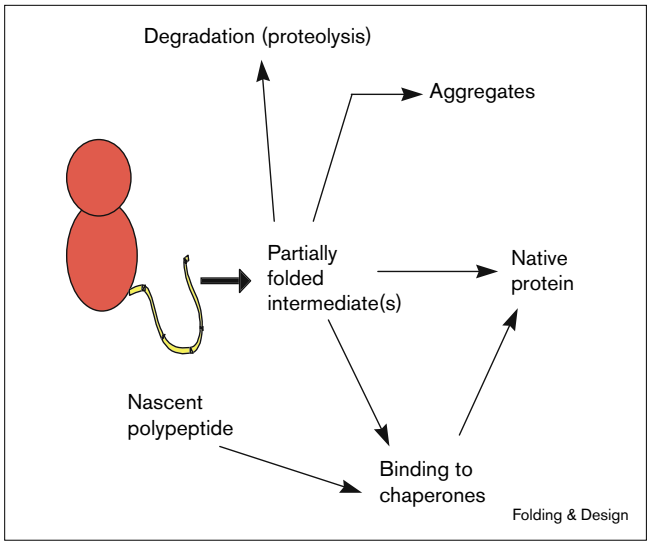
been removed [41]. Similar arguments apply to amyloid fibril formation.

Factors favoring aggregation

Circumstances that lead to the population of partially folded intermediates, especially if their concentration is high, are thus likely to lead to aggregation; these circumstances include mutations that lead to differential destabilization of the native state relative to the partially folded intermediate, as well as environmental conditions. Consequently, the major factors that determine whether a protein will aggregate, and the extent and rate of the aggregation, are: the protein amino acid sequence, the pH, the temperature and ionic strength, the concentration of the protein, the presence of cosolutes (e.g. denaturants such as urea, other chaotropes or kosmotropes — including osmolytes — and ligands that interact selectively with either the native or non-native conformations of the protein or the aggregated form), and the presence (or absence) of various molecular chaperones. In fact, a major role of chaperones is to prevent the potential aggregation of newly synthesized proteins, or those denatured through stress.

In most instances of aggregation, especially *in vivo*, there is a kinetic competition between aggregation and other processes, such as folding (Figure 4). The environmental conditions and the protein concentration significantly affect the degree and rate of intermolecular association. The protein concentration enters the equation because aggregation minimally involves a second-order kinetic process (although the growth of amyloid fibrils, and other aggregates, may appear first order). In the context of physiological aggregation, the potential role of post-translational processing may be critical. It is likely that in many cases

Figure 4



Aggregation usually involves kinetic competition. During *in vivo* protein synthesis, for example, partially folded intermediates are divided between pathways leading to spontaneous folding and the native state, aggregation, binding to chaperones and proteolytic degradation.

when aggregation occurs from a solution of the native protein it is the partially folded intermediates in equilibrium with the native state that are the immediate precursors of the aggregates.

The hypothesis that aggregation arises from partially folded intermediates explains the apparent lack of correlation between protein stability and aggregation that is sometimes observed [42]. Thus, if the decreased stability of the native state of a mutant form also destabilizes the partially folded intermediate that is responsible for aggregation then a correlation may be observed. On the other hand, if destabilization of the native conformation increases the population of a partially folded intermediate that aggregates then increased aggregation will be observed and there will be no apparent correlation with the native-state stability.

The propensity for a given protein to aggregate, either *in vivo* or *in vitro*, may well be determined in part by the lifetime of partially folded intermediates. Longer lived intermediates are more likely to lead to aggregation for two reasons: first, there is a greater chance of interaction with another such partially folded intermediate, and second, in the *in vivo* situation, the molecular chaperones involved in preventing aggregation by sequestering the partially folded intermediate may become saturated, and thus there will not be enough free chaperones available to bind to additional newly synthesized protein. Several observations indicate that *in vivo* and *in vitro* aggregation during folding give rise to similar aggregates, suggesting that it is likely that a common partially folded intermediate is responsible for both types of aggregates [26,30,43,44].

In vivo aggregates: inclusion bodies

It is noteworthy that inclusion body formation is found not only in prokaryote and eukaryote cells, but also for both heterologous and homologous overexpression. This emphasizes that it is the overexpression itself that is responsible for the aggregation. For example, *in vivo* aggregation of β-lactamase is only observed when the rate of expression exceeds 2.5% of the total protein synthesis rate [45,46]. This mirrors the observations with *in vitro* refolding systems, which show that aggregation increases as the protein concentration increases [44]. Despite the fundamental and practical importance of inclusion bodies, rather little is known about their structures and mechanisms of formation. No correlation has been found between inclusion body formation in recombinant proteins and a wide variety of factors, including size and hydrophobicity, although some correlation with average charge and fraction of turn-forming residues has been observed [47]. Inclusion bodies are frequently refractory to renaturation. To obtain functional protein requires denaturation and solubilization, often with disulfide reducing agents, and subsequent renaturation. It is frequently observed that the only way to renature significant amounts of soluble material is to use extremely low protein concentrations to avoid aggregation. As a result, inclusion bodies are a major problem in biotechnology and the development of protein drugs [48].

Although many inclusion bodies are refractile in phase contrast microscopy, some large insoluble aggregates in *Escherichia coli* are not refractile, and have been called 'floccule-type' inclusion bodies [49]. At least in some cases, these appear to be less tightly packed, and more easily solubilized than classical inclusion bodies. It is possible that these aggregates arise from native protein of very limited solubility, rather than partially folded intermediates. We have noticed that there is a range in the denaturant solubility of *in vivo* insoluble proteins: classical inclusion bodies are relatively resistant to solubilization, whereas some insoluble material is much more readily dissolved in denaturant and is clearly morphologically different (S. Seshadri and A.L.F., unpublished observations). Morphological differences have also been observed between cytoplasmic and periplasmic inclusion bodies, reflected in differences in denaturant solubility and protease resistance [50].

Several factors have been suggested to lead to inclusion body formation, including: the high local concentration of protein; a reducing environment in the cytoplasm; lack of post-translational modifications; improper interactions with chaperones and other enzymes involved in *in vivo* folding; and intermolecular crosslinking via disulfides

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