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Protein Oligomerization

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<http://dx.doi.org/10.5772/57489>

1. Introduction

Protein oligomerization is a wide and fascinating topic concerning the behavior of proteins that can form supramolecular structures, either naturally or artificially. Proteins can homo- or hetero-oligomerize through a covalent, almost always irreversible stabilization, or through often reversible associations mediated by electrostatic and hydrophobic interactions, or H-bonds. The structural and functional aspects of protein oligomerization have acquired increasing importance especially in the last two decades. The improvement of the X-ray analyses quality, and NMR potential, as well as the incoming of dynamic light scattering (DLS) or surface Plasmon resonance (SPR) techniques allowed to understand features unknown before or to correct notions that were wrongly believed true. Protein oligomerization is often a phenomenon crucial in triggering various physiological pathways. On the contrary, in different compartments other protein oligomers can be the first deleterious seed driving to protein fibrillization, an event implicated in several devastating neurodegenerative diseases. In the latter case, the isolation and analysis of the oligomeric species, considered as the real toxic agents, remained elusive for a long time. Only very recently new techniques, such as solid-state NMR, Cryo-transmission electron microscopy (Cryo-TEM), High-Resolution Atomic-Force Spectroscopy, Molecular Modeling, allowed to discover structural and functional data that can clarify the determinants of a very complicated pathway.

Under the light of these recent discoveries, the chapter aims at exploring the most important structural and functional aspects of natural or artificial oligomers.

In the first paragraph after this we introduce a tentative rationalization of the terms related to the wide world of protein oligomerization; in the second we unveil the structural and mechanistic features of the different protein oligomers that can natively or artificially form; in the third we analyze the stability of protein oligomers and the factors influencing or affecting it; in the fourth we describe the functional (benign) versus the aberrant interactions determined

by protein oligomerization; in the fifth paragraph, finally, some hints related to possible industrial applications of protein oligomers will be mentioned.

Numerous cases and literature reports related to different protein will be described, especially the ones whose oligomers have been extensively studied. In this context, ribonucleases (RNases), and ribonuclease A in particular, which have become useful and interesting models, will be considered.

2. Natural and/or artificial protein polymers: Oligomers, multimers, aggregates, fibrils; tentative definitions and classifications

Before describing and analyzing the protein oligomerization events in detail, some definitions are necessary.

First of all, proteins can self- or cross-associate either naturally or artificially. The artificial phenomenon can occur when the environmental conditions of a protein solution are changed, or cross-linking chemical reaction(s) are introduced.

If a monomeric protein, i.e., lacking quaternary structure, is considered as the starting species, we can obtain the first polymeric seed when a dimer, or different dimers, form. Then, polymerization can continue towards trimer(s), tetramer(s), up to decamer(s) and so on. All these protein species are polymers that in the literature are termed, in turn, oligomers, multimers, or large aggregates, protofibrils and fibrils. The smallest subset of different subunits forming an oligomer is the structural unit of an oligomeric protein, and can also be called protomer. A promoter can be a protein subunit or several different subunits that assemble(s) in a defined stoichiometry to form an oligomer. For example, hemoglobin consists of four subunits, two α -chains and two β -chains. The oligomer stoichiometry is thus $\alpha_2\beta_2$. Hemoglobin is a heterotetramer, but it is also a dimer of two $\alpha\beta$ -protomers. Several authors use the terms protein oligomers, multimers or aggregates, to define protein species that range between a dimer and a proto-fibril. Consequently, the reader cannot withdraw the right meaning of what is reported unless from the context in which these terms are used. These different definitions are due to the lack of a clear-cut terminology assignment to the various supra-molecular protein products forming.

Although the elegant classification of homomers recently reported by Levy & Teichmann [1] is based on the symmetry and size of the structures, i.e., the number of subunits, it does not distinguish between homo-oligomers and large homo-polymers. Thus, we introduce here a tentative rationalization of the terminologies. In addition, rules that may help to assign the right terminology are lacking also because protein oligomers can be distinguished in two different ways: a) on the basis of the number of subunits forming the polymer (i.e., on the basis of the polymerization degree) or b) on the basis of the molecular weight (M.W.) of the polymer.

These two different approaches represent the principal controversy that may generate confusion. In fact, if an oligomer can be formed by two to eight-ten protein subunits, a protein of 50 kDa can be considered oligomeric also when its M.W. reaches 400-500 kDa. Instead, a 10

kDa protein is oligomeric when it reaches a weight of 80-100 kDa. On the contrary, if the oligomeric limit were a MW of, hypothetically, 100 kDa, the former protein would be larger than an oligomer already at a trimeric status, while the latter would be an oligomer also when composed by ten subunits, thus being a decamer. Protein oligomerization is heavily involved also in the formation of amyloidogenic (or not) fibrils, and in this scenario it is difficult to find the most appropriate terms able to indicate oligomers or other supramolecular structures preceding fibrillization [2] because of the transient nature of these structures. Moreover, it is almost impossible to determine the exact number of subunits forming a fibril, although the main criterion to distinguish an oligomer from a fibril is that the former is soluble, while the latter is not.

Consequently, the M.W. classification (low-molecular/high-molecular weight oligomers) becomes prevalent in the 'fibrillization' context [3, 4], while the classification based on the polymerization degree [5, 6] can be useful in the context of natural or artificial, limited or controlled, self- or hetero-protein(s) association.

To make the story short and to tangibly classify the protein polymerization (oligomerization) events we can enter two paths, one following controlled oligomerization, the other trapping protein oligomers before undergoing fibrillization and using A β -amyloid peptide of 40-42 residues as the monomeric unit [7]. From these two different, although possibly overlapping terminologies, although referring to different structures and/or structural elements, the following classification could be adopted:

1. Limited protein polymerization (not resulting in fibers)
 - a. Oligomers: from dimer(s) to pentamer(s)
 - b. Multimers or higher-order oligomers: from hexamer(s) to pentadecamers
 - c. Very large oligomers or large multimers: from pentadecamers up to more than 20-30mers.
2. Uncontrolled and extensive oligomerization driving to fibrillization:
 - a. (A β) Pre-Fibrillar Oligomers: from a tetramer of 18 kDa to structures of about 75 kDa [7].
 - b. (A β) Fibrillar Oligomers: from a dimer or a tetramer of 9-18 kDa to structures of about 500 kDa [7].
 - c. Proto-fibrils: structures lacking order and periodic symmetry of mature fibrils; shorter and less linear than fibrils [8], and often deriving from 'globulomers' [9] or 'annular oligomers' [10], already called 'ring-like shaped' annular aggregates [11].
 - d. Fibrils: ordered, symmetrical, long, varying in length, extending towards several micrometers [12], stacked filaments of unbranched cross- β -spine [13] pairs, ranging from about 60 up to 200 Å in diameter or width [8, 14].

Thus, different types of oligomers deriving from the same protein or domain can certainly overlap in terms of size and M.W. Consequently, it has to be also taken into account that the

same protein can be considered as to be fibrillogenic or not fibrillogenic not only on the basis of its dimensions or M.W., but also because of its morphology, toxicity, pathway of formation, or method of artificial formation of its oligomers [2, 7, 8]. Furthermore, these supramolecular species can be toxic or harmless provided they satisfy, or not, some requisites [2, 15] that will be discussed in the following paragraphs.

Based on what it has been reported, the terms oligomers or others related to supramolecular protein structures may be still somehow misleading and equivocal. Anyway, the tentative classification here proposed and based on two different contexts, i.e., fibrillogenesis or not, will be used in the following paragraphs.

3. Protein oligomers

3.1. Homo/hetero-dimeric or oligomeric proteins

Several studies have been focused, especially in the last two decades, on the features of homopolymeric or, more precisely, homo-oligomeric proteins, i.e., supramolecular structures formed by self-associating proteins. In this context, a great amount of structural, mechanistic, physicochemical, and functional elements have been discovered and elucidated as important determinants which tune and control protein self-association.

Instead, except the very well known heterotetrameric hemoglobin, less is known about hetero-oligomeric protein complexes. These hetero-structures refer to chains of different sequences, which undergo association pathway in a way less statistically favorable and easily controllable, either qualitatively or quantitatively, than protein self-association. Nevertheless, protein hetero-oligomerization represents a very important phenomenon in the formation of molecular machines, like for motor proteins (kinesin, microtubules,), or, alternatively, can be obtained artificially by the use of asymmetric bifunctional reagents, while new discoveries of natural hetero-protein association events lighted up again the interest over this topic.

3.2. Covalently linked oligomers

Although not very frequently, protein cross-linking can occur naturally forming covalently linked species that display quaternary structures, or active covalent complexes starting from inactive monomeric precursors. Post-transduction modifications, photochemical event(s), or co-enzyme binding (i.e., going from apo-to holo-forms), can also induce protein self-or cross-linking.

Natural cross-linking can sometimes occur through free cysteines of two different subunits that can couple to form intermolecular disulfides depending on the redox state of the environment. This is the case, for example, of bovine seminal RNase (BS-RNase), the unique member of the large pancreatic-type secretory ribonuclease super-family which is dimeric in nature [16]. Other proteins that can form covalent supramolecular structures are structural proteins such as collagen or elastin, the latter forming or disrupting in tissues or vessels desmosine bridges forming between lysine residues after elastic stress or relaxation [17].

Alternatively, artificial cross-linking(s) can produce oligomers after the reaction of a protein with dehydrating molecules, such as EDC or carbodihymines in general, or through the use of several bifunctional reagents, such as dialdehydes or diimidoesters [18, 19]. These two latter chemicals display two terminal reactive groups separated by a variable number of unreactive spacers, such as methylenes. They are often symmetric, but sometimes asymmetric. The alternative use of them becomes advantageous depending on the goal to be reached, as for example for a homo- or hetero-protein cross-linking, respectively.

Some of these different reagents were extensively used already in the late '50s and '60s to produce protein oligomers that have been useful, after limited proteolysis, to study protein primary and tertiary structures, and, thus, protein conformations. Later, the formation of covalently linked oligomers allowed sometimes the production of protein derivatives with increased activity, and also higher stability against proteases, and so on.

One of the first class of reagents used in protein cross-linking were dialdehydes, such as glutaraldehyde (Figure 1 a) or diimidoesters [19], like dimethyladipimidate, dimethylpimelimidate and dimethylsuberimidate (Figure 1 b).

Both classes of these cross-linkers react mainly with lysine residues, which act as nucleophiles towards the aldehydic or imidic carbon of the cross-linker (Fig. 1 c) under slightly basic environmental conditions (pH ranging from 7.5 to 8.5-9). By this reaction a yield of more than 20% of dimers can be obtained, as well as a decreasing amount of trimers, tetramers and traces of higher-order oligomers.

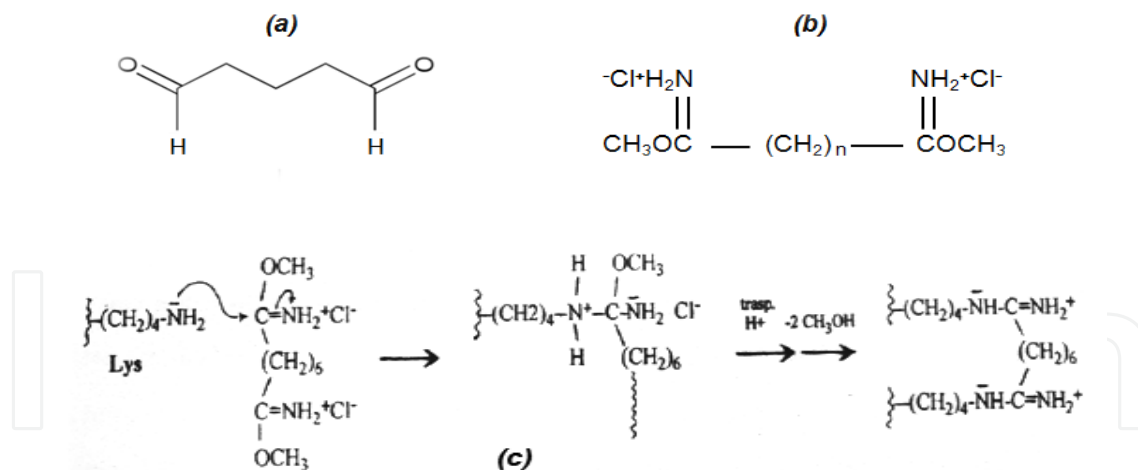


Figure 1. (a) Glutaraldehyde. (b) Diimidoesters: n=4, dimethyl-adipimidate; n=5,-pimelimidate; n=6,-suberimidate. (c) Mechanism of reaction of a protein with an imidoester (dimethylsuberimidate).

The advantage represented by diimidoesters with respect to dialdehydes, like glutaraldehyde, is that the latter are toxic, generally more reactive than diimidoesters and consequently often able to introduce unwanted changes in a protein. Moreover, sometimes they strongly drive towards side intramolecular reactions with a possible inactivation of the protein. Diimidoesters reactivity, on the contrary, can be better controlled although protein lysines may be modified,

and allows to maintain the overall charge of the protein unmodified. Consequently, despite the oligomeric yields obtained with diimidoesters are lower than those of dialdehydes, the products obtained are more specific and active than the ones obtained with the latter. Finally, the longer the spacer, the higher the intermolecular yield and the less rigid oligomeric product obtainable [19]. Thus, dimethylsuberimidate is more useful to artificially oligomerize a protein than the other shorter diimidoesters mentioned above.

Other reagents that can be useful to obtain protein oligomers are the bifunctional N-substituted maleimide derivatives [20], such as *N,N'*1,2- or 1,3- or 1,4-phenyldimaleimide (ortho-meta-para-PDM) (Figure 2 a-c), or derivatives displaying spacers of varying length between the two reactive maleimides (Figure 2 d-f). The reaction occurs between the maleimide and the free sulfhydryl group of a cysteine of the protein as an addition which saturates the double link of the maleimide conjugated with two carbonyls (Figure 2 g) [20].

The cross-linking event can be irreversible or reversible. Reversibility can be favored, for example, by the use of spacers containing disulfide bonds (Figure 2 g). These “redox” spacers can be applied to all bifunctional reagents displaying spacers if the reversibility of the reaction is desired, and are very tricky for asymmetric bifunctional linkers.

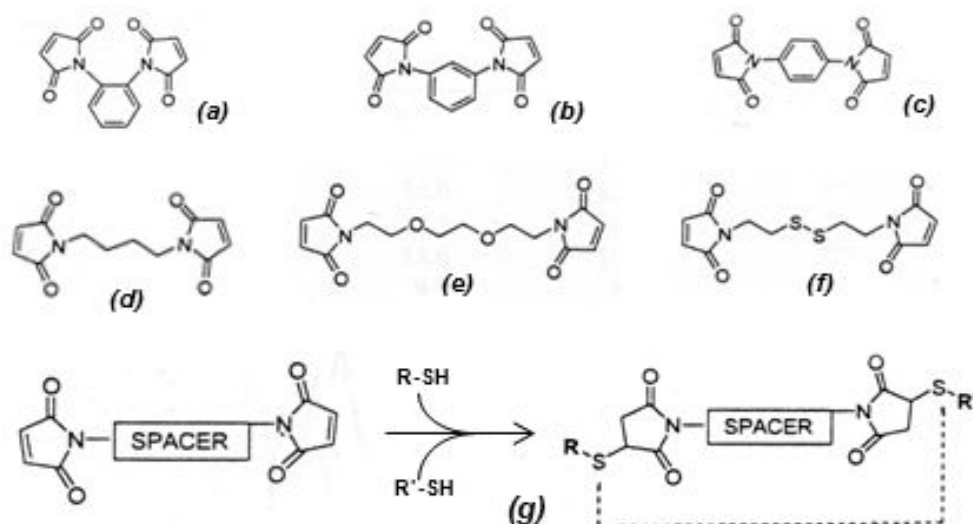


Figure 2. Some bifunctional maleimides.(a), (b), (c) ortho-meta-para *N, N'*phenylenemaleimide (o,m,pPDM); (d) 1,4-bismaleimidobutane (BMB); (e) 1,8-bismaleimidotriethyleneglycol (BM[PEO]₃); (f) dithio-bis-maleimidoethane (DMTE); (g) Reaction of a bis-maleimide derivative with sulfhydryl groups (free, reduced cysteines). Figure adapted from [20].

This can allow, for example, to obtain species that need to be driven inside cells as heterodimers and then to be released as dissociated monomers exploiting the reducing environment of the cytosol. Thus, spacers containing disulfides can be applied to all bifunctional reagents if the reversibility of the reaction is desired.

Asymmetric bifunctional reagents can be very useful to covalently link antibodies or part of them (light/heavy chains) with proteins, protein domains, or toxins, or whatever of biological interest. They can be a combination of maleimides or succinimides displaying or not dithio-

derivatives in the spacers, and coupled, in the second terminus, with imidoesters, diones, thiones, 2-iminothiolane, etc. Almost all of these bifunctional cross-links foresee a two-step reaction in which one of the partners is firstly modified and activated (for example, with 2-iminothiolane) in order to become able to react with the appropriate partner and form the new hetero-dimeric adduct. Some of the most important reactants used for artificial heterodimerization are 1-(3-((2,5-Dioxopyrrolidinyl)oxy-carbonyl)phenyl)-1H-pyrrole-2,5-dione (MBS) N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), S-4-succinimidyl-oxycarbonyl- α -methyl benzyl thiosulfate (SMBT), or 4-succinimidyl-oxycarbonyl- α -methyl- α -(2-pyridyldithio)toluene (SMPT). The details of these hetero-oligomerization reactions are beyond the aim of this chapter but are clearly described in the review of Fracasso and colleagues [21].

Again, also another dione, 4-phenyl, 1,2,4-triazoline-3,5-dione (PTD), structurally similar to maleimides but containing nitrogens in the pentagon ring, can be useful to covalently cross-link proteins through amino-groups [22].

Other important bifunctional reagents are carbodiimides ($R_1-N=C=C-R_2^+$), such as EDC, which exploits its dehydrating power to create new isopeptide bonds between the side chains of Lys and Glu or Asp residues of proteins [23], thus producing a “zero-length” cross-linking. This reagent can be very useful to covalently fix previously formed oligomeric protein aggregates [24] and allows to avoid unwanted insertions of chemicals or net charge modifications in the protein complex.

Divinyl sulfone (DVS) [25] and dinitrodifluorobenzene (DFDNB) [26] are two bifunctional reagents that deserve to be mentioned (Figure 3). They lack spacers, and to stabilize preformed structures without affecting oligomers' conformations [25, 27-29].



Figure 3. (a) Divinyl sulfone (DVS). (b) Dinitrodifluorobenzene (DFDNB)

DVS is specific for histidines, while DFDNB reacts with lysines. The limited dimensions of both molecules allow cross-linking only between residues that are very close to each other. For this reason subsequent chromatographic or electrophoretic analyses can highlight important conformational features of the protein or its oligomers. The cross-linking can drive towards intra-or inter-molecular adducts, thus revealing if the protein was monomeric or already oligomeric before the covalent stabilization [25].

Finally, covalent protein “zero-length” oligomerization can be obtained, without chemicals, by sealing a lyophilized protein under vacuum at high temperature, up to 85 °C, for 24-96 hours [30]. This process permits to obtain dimers, trimers, and traces of tetramers of ribonuclease A (RNase A) and lysozyme, without the introduction of chemical groups which could

totally or partially inactivate the protein residues involved in the reaction. In fact, the heat-vacuum treatment of proteins induces the dehydration of some of the Lys and Asp or Glu side-chains, thus producing newly formed intermolecular isopeptide bonds. However, this reaction, firstly considered to be specific of a single couple of Lys and Glu of RNase A [31], was later found to involve more than one of these acid or basic residues, thus producing a mixture of heterogeneous products [32].

Last, but not least, cross-linked oligomers can be produced also through UV photochemistry. The UV-treatment of a monomer or pre-formed non-covalent oligomers [33] produces covalently stabilized oligomers. If the photochemical treatment inactivates the native protein or its possibly pre-formed oligomers, structural important information can be anyway withdrawn from these artificial modifications [34, 35].

3.3. Non-covalent protein oligomers

Protein association can occur very often naturally, and without covalent modification(s), through a homo-or hetero-association mediated by a weak-bond-network, formed by electrostatic or hydrophobic interactions, and/or specific H-bond(s). If the interface between monomers or protomers is large almost all types of interactions can occur and are more frequently conserved. Small interfaces have been probably acquired recently in evolution [36]. These interactions can be crucial for the active forms of several class of proteins, such as enzymes and transporters. These interaction(s)/association(s) may occur naturally because of the sequence and structural features of the subunits which build the oligomeric complex(es). Otherwise, they can form because of environmental changes, like pH or ionic strength, or as a consequence of the increase of the monomers local concentration.

These events allow the protein to overpass its dimerization dissociation constant(s) $K_{D1/2}$ (equation 1 and Figure 4, red and orange).

$$K_D = \frac{[M]^2}{[D]} \quad \text{with } M = \text{monomer}, D = \text{dimer} \quad (1)$$

A further increase of the concentration can augment the degree of polymerization with the formation of trimers (Figure 4, green pictures), tetramers (Figure 4 magenta, pink, violet), and larger oligomers or multimers if the entropy cost is balanced by favorable interface interaction(s).

The free energy of multimerization is reported in equation 2 [37].

$$\Delta G = \Delta G^0 + RT \ln \left(\frac{[nP]}{[P]^n} \right) \quad (2)$$

in which [P] is the concentration of protein segment(s) exposed, or protein interfaces [37], and prone to interact with other protomers.

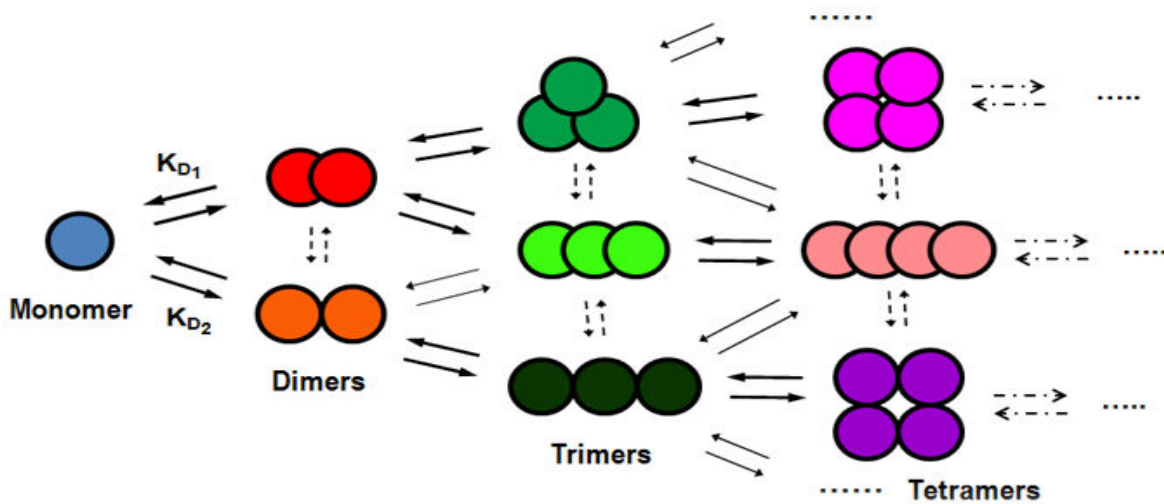


Figure 4. Schematic view of protein oligomerization. A native monomer (blue) can become a dimer if K_D of dimerization (equation 1) is overpassed. Two possible dimeric conformers with different interfaces or interface areas are shown to form with K_{D1} and K_{D2} respectively. These events can lead to the formation of different trimers (three green conformers are shown), tetramers (magenta, pink, violet) and so on. Trimers and tetramers unlikely form from isolated monomers because of entropic reasons, but can grow from the pre-existing oligomer(s) (or also from two dimers). Oligomers can follow different pathways (dotted lines) to form higher-order oligomers or multimers, and can undergo conformational rearrangements which can compensate the entropy cost to be payed to associate the subunits.

An example of natural oligomeric protein is represented by the oxygen transporter hemoglobin (Hb), which is functionally active only as an $\alpha_2\beta_2$ tetramer (or, in the fetus, $\alpha_2\gamma_2$, endowed with a greater affinity to oxygen) with its subunits being associated through salt bridges and other weak interactions. The loss of these interactions drives Hb to switch its conformation from the deoxygenated (tense, T) to the oxygenated (relaxed, R) form, and this modification is due to allosteric interactions triggered by the first oxygen bound (Figure 5). Hb, unless being tetrameric, cannot be active, while myoglobin, the oxygen collector in tissues, is active as a monomer.

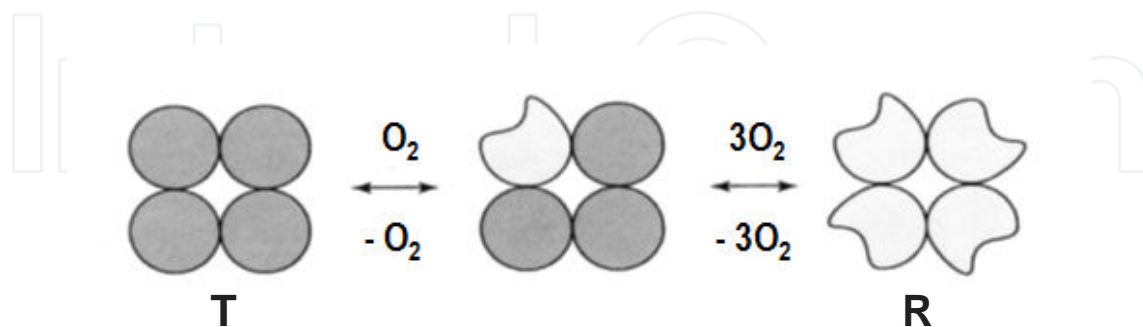


Figure 5. Allosteric control of haemoglobin (Hb) induced by oxygen binding.

Another important oligomeric protein transporter is transthyretin (TTR), formerly called pre-albumin, one of the transporters of the hormone thyroxine and of the lipocalin retinol-binding protein (RBP), the specific carrier of A-vitamin [38]. TTR is natively a 55-kDa dimer of dimers,

or homotetramer, mainly composed of β -sheets [39]. Its peculiarity consists of the pathologic pathway it follows if destabilized by malignant mutations: if so, monomers dissociate from tetrameric assembly and undergo uncontrolled aggregation and fibrillization through the formation of intermediate annular oligomers [10]. These findings will be further discussed below.

Other cases of non-covalent association between proteins can be represented, for example, by 5' pyridoxal-phosphate (PLP) enzymes, such as the aspartic aminotransferase (AAT), alanine-glyoxylate-aminotransferase (AGT) (Figure 6a), dopa-decarboxylase (DDC) (Figure 6 b), and cystalysin (a lyase, like DDC). Inactive as monomers, these enzymes are active only when they are in form of dimers, although a detailed analysis of their dimerization pathways has been performed only with *Treponema Denticola* cystalysin mutants [40].

The family of the PLP-dependent enzymes is very large, forming five different fold types, and some members of the family are active as tetramers or hexamers. AGT [41] and DDC [42], shown in Figure 6, are dimers belonging to Fold Type I, as well as cystalysin and AAT. Belonging to Fold Type I means that each subunit of the holo-form host a PLP molecule, but the active site is composite, i.e., formed by residues of both subunits. Instead, enzymes belonging to Fold Type II are active as dimers or oligomers binding one PLP each at the same time, but they evolved to form one active site per subunit, and are often accompanied by the presence of allosteric regulation domains.

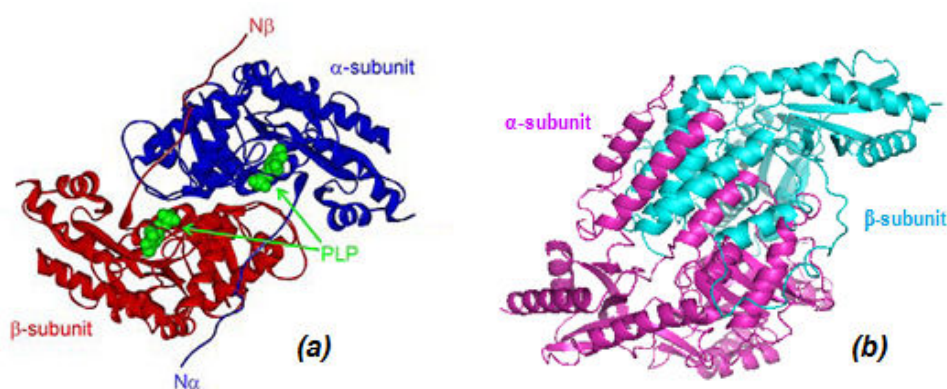


Figure 6. Three dimensional structure of holo natively dimeric (a) human liver alanine-glyoxylate aminotransferase (hAGT) [41] (b) pig kidney dopa decarboxylase (pkDDC) [42] (Burkhard P. *et al* 2001, *NSB* **8** (11) 963-7). The two α/β subunits are shown with different colours and for AGT the location of the two PLP molecules is shown in green. AGT is peculiar for the wrapping of each terminal arm ($N\alpha/N\beta$) into the region occupied by the complementary subunit.

This evolution pathway allowed them to build domains which are active as allosteric regulators. The association of the subunits of these PLP-enzymes is often mediated mainly by hydrophobic interactions because the monomeric forms are readily prone to uncontrolled extensive aggregation [35]. Anyway, the inter-subunit surfaces being often very large, several types of contacts occur, such as electrostatic interactions or H-bonds.

Another interesting example of oligomeric protein is represented by mammalian Phenylalanine Hydroxylase (PAH), which is a homo-tetrameric enzyme made of four 50 kDa subunits

whose overall 3D structure has not been solved yet. Its activity depends on its tetrameric structure and the presence of tetrahydrobiopterin (BH₄), and is allosterically regulated by the substrate itself (Phe). Some mutations become pathogenic because they destabilize and inactivate the tetramer, and consequently drive the organism towards the incoming of Phenylketonuria (PKU) [43].

A natively oligomeric protein can also switch towards higher-order oligomers. Indeed, the natively homotetrameric L-rhamnulose-1-phosphatase aldolase becomes an octamer only upon a single A88F mutation [44]. The introduction of a single residue displaying large non-polar side-chains (Phe, Trp) can be sufficient to drive the native oligomer towards larger multimeric complex(es).

It has to be mentioned here that from a total of about 450 well characterized enzymes, only about 140 of them are monomeric. Of the other 310, 200 are homo-oligomers/multimers and in detail: 125 homodimers, 50 homotetramers and 25 are structures larger than tetramers. Finally, the remaining about 110 are hetero-oligomers/multimers [45].

Protein oligomerization can also be a non-native event: indeed, natively monomeric proteins can naturally and non-covalently undergo oligomerization as a sort of post translational event which can become a switch between active and non active products. This is true, for example, for several trans-membrane receptors, which often display kinase activity. Upon ligand binding, the intracellular domain dimerizes, this event triggering (auto)phosphorilation of the intracellular domain which undergoes conformational changes and is able to activate a signal transduction cascade that induces or tunes important physiological phenomena. Examples of families of this type of receptors are: growth hormone, interferon, cytokine and Tyr-kinase, G-protein-coupled receptors families (GPCRs) [46]. The components of the latter family were initially thought to act as monomers, but several pharmacological, biochemical and biophysical data indicate that GPCRs function as cooperatively controlled dimers [47].

Another very interesting example is represented by the Caspase-3,-7, and-9, a family of proteins involved in apoptosis. Under physiological conditions Caspase-9 exists as an inactive monomer forming an 1:1 complex with the Apaf-1 cofactor in the presence of Cytochrome C and ATP to produce a heteromultimer. This complex co-localizes with a multiple array of Caspase-9 molecules, which consequently increase their concentration above the Caspase-9 homodimer dissociation constant K_D . This event allows the homodimer to be formed through the exposure of an activation loop, and the active dimer provides the catalytic activity necessary to activate Caspase-3 and-7 [48].

Finally, other interesting examples of oligomeric natural proteins are the membrane channel-forming tetrameric complexes that allow specific ions (Na⁺ or K⁺) or water to permeate cells, such as aquaporins or aquaglyceroporins [49].

Proteins can also form large pathogenic oligomers or multimers that can evolve towards pathogenic supramolecular structures. Important examples of these malignant events are the uncontrolled aggregation of the Glu6Val Hb mutant of Hb (E6V-Hb or HbS) in sickle cell anaemia or the formation of amyloid or amyloid-like fibrils, as it occurs with several proteins related to severe neurodegenerative diseases. These latter products, overpassing the oligo-

meric status, will be discussed later in a greater detail, within the physio-pathological functional consequences of protein oligomerization.

3.4. Self/cross-association through three dimensional (3D) domain swapping

A peculiar, interesting way to form protein dimers, oligomers or large multimers can occur naturally or artificially through the reciprocal exchange of small or large regions (peptide(s) or entire domain(s)) of the monomeric subunits. Monomers exploit short flexible hinge-loops present in their sequence to address a definite domain (or more than one) into the corresponding partner subunit that will reciprocally swap an identical domain with the former (Figure 7). This mechanism was called three dimensional domain swapping (3D-DS) by Eisenberg and co-workers when they discovered that diphtheria toxin (DT) can form a dimer intertwining an entire domain [50]. Beyond dimers, this mechanism, known for DT as well as for other proteins, can also lead to the formation of larger oligomers [51], exploiting a small flexible loop (Figure 7) that is able to adopt different conformations within various different environmental conditions.

The domain-swapped oligomer reconstitutes the native contacts present in the monomer (closed interface [52], green in Figure 7) except the hinge loop, while a new interface (open interface [52], magenta in Figure 7) forms in the oligomer only, stabilizing it.

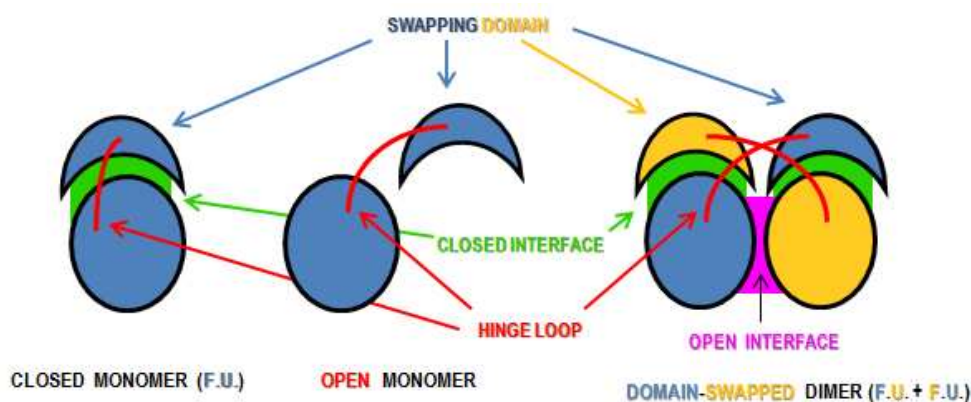


Figure 7. Schematic view of the 3D domain swapping (3D-DS) mechanism. The movement opening of the loop present in the 'starting' monomer (blue) allows the formation of the dimer by recreating the intramolecular interdomain interface present in the monomer (closed interface, green [52]) in an intermolecular dimeric interface instead. This subsequently drives the formation of a new dimeric interface (open interface, magenta [52]), absent in the monomer. Through this mechanism a protein can form an active dimer still maintaining functional units (F.U.). The same mechanism can drive to oligomers of higher stoichiometry (number of associated chains) than dimers. (Modified from [53]).

3D domain swapping (3D-DS) was hypothesized about fifty years ago [54] for RNase A, and was then confirmed by several brilliant crystallographic results obtained in the '90s, the first with BS-RNase [55]. In the last two decades, domain swapping has been discovered to involve more than sixty proteins [56], and about 300 domain-swapped structures have been solved in crystals or solution, while even a higher number of models of oligomeric swapped proteins

have been proposed. Furthermore, 3D-DS is almost uniquely related to protein self-association, although rare cases of hetero-association exist.

Eisenberg and co-workers, who named 3D-DS this mechanism of reciprocal exchange of domains between proteins, immediately underlined the double-face nature, malignant and/or benign, of this protein-protein interaction, defining it as “entangling alliances between proteins” [50]. The term “entanglement” can be forced to be considered as expressing a negative fate of a protein without any possibility to evade the interaction although this ‘jailing’ can not be known ‘a priori’. This is the case of amyloidogenic proteins which form, through this mechanism, fibrils involved in neurodegenerative diseases. Conversely, the term “alliances” clearly indicates the benign face of 3D-DS. In fact, a protein can self-associate to acquire novel activities absent in the monomer, or could also enforce pre-existing ones [57, 58], or control them, for example allosterically, as it occurs for swapped dimeric RNases [59, 60].

The mechanism is made possible thanks to the presence of a hinge-loop (red in Figure 7) located between two different protein structured domains. The flexible loop changes its conformation depending on the environment, and can address the swappable domain(s) into the complementary subunit(s). The parts of a protein to be exchanged can be elements of secondary structure, such as α -helices or β -sheets (RNase A, BS-RNase), or entire domains (DT). The loop is usually composed of few residues which can be different from one protein to another. Interestingly, a single point mutation can induce dramatic changes in the loop flexibility and switch the protein towards or against self association through 3D-DS [61-64]. Other important factors that govern the 3D-DS event(s) are obviously protein concentration and inter-molecular interactions [65, 66].

Some proteins can be constitutively domain-swapped, as it is for the member of the cyclin-dependent kinase p13suc1, which is natively a mixture of a monomer and a domain-swapped dimer [67]. The two monomeric/dimeric states are in equilibrium and the domain-swapped dimer is favored by the presence of proline residues in the hinge loop [68].

Another very interesting natively domain-swapped protein is one of the two conformers of BS-RNase (Figure 8). This protein is the unique natural dimer of the large pancreatic-type RNases superfamily, whose proto-type is RNase A. Furthermore, BS-RNase is a mixture of two isoforms [60], which are both covalently dimeric because of the presence of two antiparallel disulfide bonds, i.e., occurring between Cys-31 and-32 of one subunit and Cys-32 and-31, respectively, of the other [69]. About 70% of the molecules spontaneously swap their N-terminal helices to form the conformer called MxM shown in the right panel of Figure 8. This swapping event implies interesting functional consequences that will be discussed later. Thus, About 30% of BS-RNase is dimeric only thanks to the cited disulfides and called M=M [60, 70] (Figure 8, left panel), while about 70% of the molecules additionally swap their N-terminal helices to form the second BS-RNase conformer, called MxM (Figure 8, right panel) [55, 60]. This swapping event implies interesting functional consequences that will be discussed later.

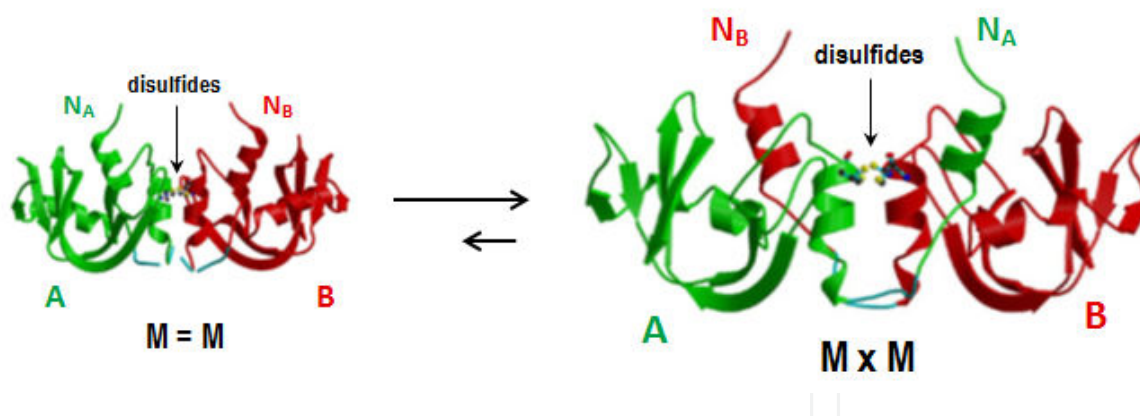


Figure 8. Structure of the two dimeric conformers of BS-RNase: left panel, the unswapped (M=M) conformer [70]; right, the swapped isoform (MxM) [55]. The two subunits, A and B, and their N-termini, are highlighted, as well as the disulfide bonds that covalently link the two subunits in both isoforms [60].

The BS-RNase hinge loop and open interface have been extensively studied [64] and, while Pro 19 and Leu 28 are key-residues for the stability of the MxM isoform [65], the entire 16-21 loop mutation together with a R80S mutation dramatically inverted the swapping tendency of the protein [63].

Other proteins, being native monomers, can be induced to form domain-swapped oligomers either naturally or artificially. This is the case of the 13.7 kDa RNase A (Figure 9 A). This enzyme can form various non-covalent oligomers when is lyophilized from 40-50% acetic acid solutions, and was the first protein for which 3D-DS mechanism was hypothesized to occur through the swapping of its N-terminal ends [54]. In 1998 this idea was confirmed by the analysis of the crystal structure of the N-term-swapped dimer of RNase A [71], now called RNase A N-dimer, or N_D [5] (Figure 9 B). Three years later, the crystallographic structure of another dimeric conformer of RNase A was solved, discovering that the protein also swaps its C-terminus to form a second dimeric conformer [72], then called the C-dimer or C_D [5] (Figure 9 C).

Several RNase A oligomers larger than dimers have also been found to form [6, 29, 73, 74]. Among them, three trimers (Figure 9 D-F), and six tetrameric different conformers have been found and extensively or partially characterized (Figure 9, panels G-N) as well as several other larger multimers [6, 27, 29, 73-75].

These findings indicate that the folds of RNase A are highly versatile, despite its overall known stability. Its dimers are not exclusively artificial, given that traces of N_D are present in a native mixture [77, 78], and that C_D has been detected to form and be subsequently degraded during protein expression in cells [79]. The capability of this enzyme to swap both termini definitely increases the number of possible structures it can form: in fact, the linear, or quasi-linear but not cyclically-closed oligomeric structures reported in Figure 9 (panels D and G-M), can form thanks to the contemporary swapping of N- and C-termini [6, 27, 28, 75, 76].

The capability to swap multiple domains highly increases the 'swapping capacity' (SC), which is defined as the upper limit of subunits with which a protein may interact [80]. In fact, if a

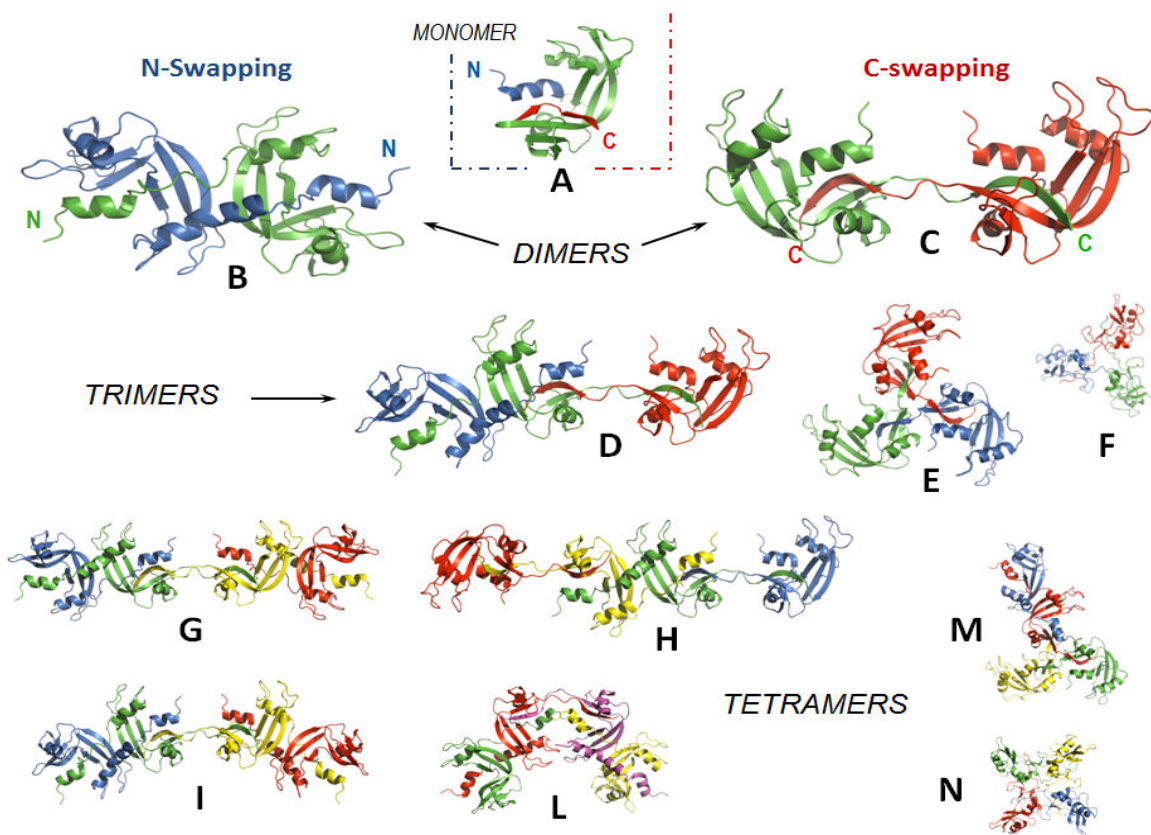


Figure 9. RNase A 3D domain-swapped oligomers. **A**, native RNase A monomer; **B**, N-dimer, N_D [71]; **C**, C-dimer, C_D [72]; **D**, N+C-swapped trimeric model [27, 75]; **E**, cyclic C-swapped-only, trimer, [75]; **F**, cyclic C-swapped-only, trimeric model [75]; **G-N**: tetramers, all N+C-swapped [6, 27, 28, 76], except the cyclic C-swapped-only model of panel **N** [76]. The dimensions of the oligomers here reported are tentatively representing the relative proportions withdrawable after the lyophilization from 40% acetic acid solutions. RNase A also forms pentamers, hexamers [6, 29, 73], and larger multimers, up to tetradecamers [6, 74], not shown here.

protein displays N swappable domains, SC is defined to be equal to N^2+N [80]. Thus, if $N=1$, then $SC=2$ ($1+1$), while if $N=2$ SC speeds up to 6 ($4+2$), and so on. RNase A was thought to be unique in its capability to swap more than one domain, but recently also BS-RNase [81], cyanovirin-N and the mammalian DUF59-Fam96a protein displayed a similar multiple DS behavior [82, 83]. In particular, the natively N-swapped dimeric BS-RNase, known to be able to self-associate since 1969 [57], was found to form either N-and C-swapped tetramers and multimers [81, 84]. In addition, it has recently been reported that a monomerized BS-RNase [85] can be induced to form a C-swapped dimer similar to the one of RNase A [86].

Furthermore, RNase A was firstly thought to be induced to oligomerize through an initial only partial denaturation [72] via 40% aqueous acetic acid treatment [54], while later it was shown that the protein undergoes almost complete denaturation (except its four disulfides) under acidic conditions [87]. Then, when lyophilization is followed by a re-dissolution of the powder in 'benign' buffers [87], i.e., buffers that can slow the regression towards monomer, as phosphate does for RNase oligomers, the protein can re-gain its native monomeric form for about 70% of the initial amount, and the remaining 30% forms various domain-swapped oligomers. The debate between the theories based on a partial [88], or total [87] denaturation

pathway that a generic protein must follow to oligomerize through 3D-DS is still open, and what has been established for RNase A [87] is not possible to be absolutely stated for all proteins.

RNase A and BS-RNase can oligomerize following the same 3D-DS mechanism also if very highly concentrated water-alcohol solutions of the enzyme are heated up to 60 °C, and then stabilized by phosphate buffers avoiding the lyophilization step [78, 81]. In this way, the absolute and relative amounts of the various N- or C-swapped oligomers change depending on the environmental conditions. Point mutations which modulated the hydrophilic/hydrophobic nature of the N-/C-swappable domains of RNase A confirmed this tendency [89].

Also human RNases like human pancreatic RNase (hp-RNase) can spontaneously dimerize through 3D-DS when some point mutations are introduced [90-92], although no oligomers larger than dimers have been detected among its aggregation products.

The 3D-DS mechanism can let a protein to overpass the dimeric status and go towards larger oligomerization, multimerization and possibly fibrillization through multiple swapping and/or other alternative ways to stabilize the aggregates. For instance, the formation of disulfide bonds, or the repeat for several times of the same type of swapping with the formation of open-ended structures through a propagative or runaway 3D-DS [93, 94] (Figure 10) are proper alternative ways.



Figure 10. DS in not-cyclic oligomers larger than dimers. (modified from [94]). The first two models display open-ended edging subunits. The increase in stability of an open-ended structure is proportional to the number of subunits that are present between the two edges. The three models reported are an evolution of the ones reported in [93].

Beyond RNases, several other proteins involved in important biologic processes show to form domain-swapped oligomers. One domain swapping-prone protein which in the last years has been discovered to be highly structurally versatile is cyanovirin-N, an 11 kDa protein that inhibits HIV [95]. It can be active either as a monomer or as a metastable domain-swapped dimer [96]. Interestingly, some mutants that become active only as domain-swapped dimers were recently found to form two different relatively stable 3D-DS dimeric conformers, one 3D-DS trimer, and two 3D-DS tetramers [82].

Other important examples of proteins able to form domain-swapped structures are the following: i) Cytochrome C, which was known to polymerize since 1962 [97], but only recently showed to form these inactive supramolecular structures protomers via a runaway 3D-DS of its C-termini. In particular, domain-swapped dimers, trimers, tetramers and polymers up to ~40-mers have been characterized [98]; ii) BCL-X_L, an anti-apoptotic protein belonging to the BCL-2 family which can form active C-term-swapped dimers when highly concentrated [99] or alternatively when heated up to 50 °C [100]. iii) Cadherins, which are cell adhesion proteins, dimerize through β -strand swapping to mediate the adhesion itself [101]. Incidentally, it has to be mentioned that several protein cell receptors are known to dimerize to become active (see above), but less is known about the mechanism responsible for the dimerization. Thus, possibly some of them could undergo 3D-DS. iv) Finally, also histones are known to fold through 3D-DS in their evolutionary pathway [102].

3D-DS can also be favored, or hindered, by point or multiple mutations, as is well known for hp-RNase, or by the conditions under which the crystallization process occurs. This is true for barnase, a 12kDa RNase from *Bacillus amyloliquefaciens*, which forms a DS cyclic trimer under not too harsh conditions [103], and for the DS dimer formed in crystals by Grb2-SH2 domains [104].

Last, but not least, some amyloidogenic proteins form fibrils through the initial formation of domain-swapped dimers and oligomers, which are the starting point of their massive self-association [80]. The possibility to overlap 3D-DS with the mechanism of formation of amyloid fibrils was firstly hypothesized by Eisenberg and colleagues. They explained it as a compatibility existing between 3D-DS and the polyglutammine(polyQ)-cross- β steric zippers [72]. This idea was supported by the structural similarity existing between the Asn-based open interface of RNase A C_D [72] and the fibrillogenic nature of poly-Q expansions [105] which are structured as cross- β -spines [13]. The validity of this theory was also enforced by the discovery that either prion protein or cystatin-C, two amyloidogenic cross- β -spine-prone proteins, form 3D-DS dimers [106, 107] (Figure 11, panels A and B). Later, numerous different experimental evidences confirmed this hypothesis. In fact, after discovering that the prion protein (PrP), which is associated with the lethal neurodegenerative Creutzfeldt-Jacobs Disease (CJD) and Scrapie, dimerizes through 3D-DS [106], the dimerization event was shown to be the rate-limiting step in the conversion towards the infectious fibrillogenic form of PrP [108]. Then, conversion to fibrils is promoted by an unlocking of the globular domain combined to a redox process, both triggered by 3D-DS [109, 110]. These events drive to the formation of domain-swapped oligomers and multimers stabilized by intermolecular newly formed disulfide bonds [110], but do not affect the overall tertiary structure of the globular main domain of PrP [109] (Figure 11 A).

Furthermore, cystatins, a class of proteins which comprises also stefins and that inhibit cysteine proteases, can also dimerize through 3D-DS [113]. In particular, the L68Q human cystatin-C (hCC) mutant in particular, can dimerize through 3D-DS [107], then inducing severe massive amyloidosis in brain arteries and lethal cerebral hemorrhages. The 13.3 kDa hCC forms fibers through a preliminary domain-swapped dimer+dimer tetrameric rearrangement [114] and a

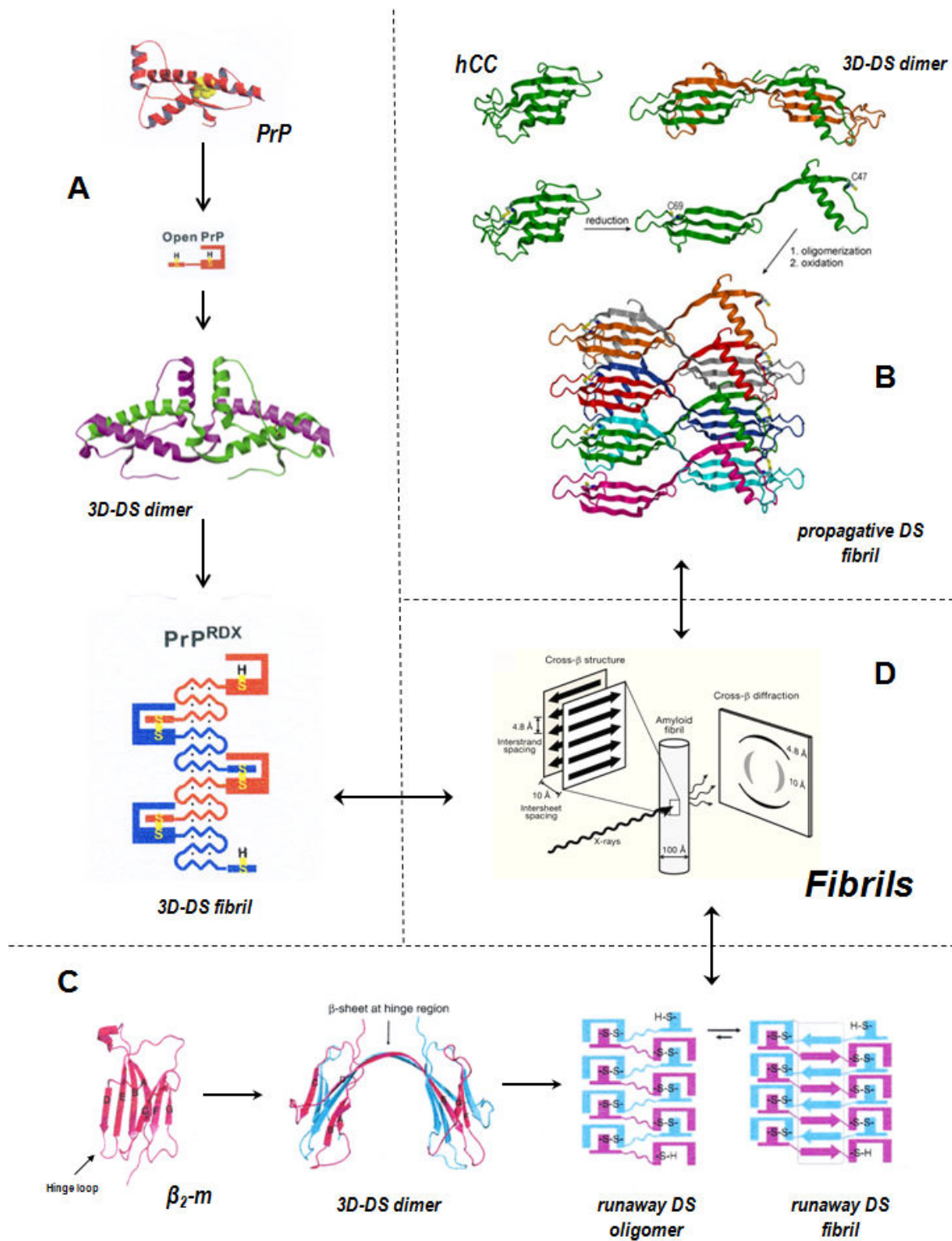


Figure 11. DS Amyloid fibrils of (A) human prion protein (hPrP), (B) human cystatin-C, hCC, and (C) β_2 -microglobulin (β_2 -m). In all cases, fibrillogenesis is promoted by the preliminary formation of a domain-swapped dimer which evolves towards fibrils through a redox pathway. All the three fibers display the features of a generic amyloid fiber shown in panel D. The figure summarizes pictures reported in [106, 107, 110-112].

subsequent propagation of 3D-DS [111]. The finding that prevention of 3D-DS inhibits cystatin-C dimerization and multimerization [115] and studies on the hinge-loop governing the 3D-DS event [116] confirm that 3D-DS plays a key-role in cystatin-C fibrillogenesis [117] (Figure 11 B).

Another important amyloidogenic protein able to self-associate through 3D-DS is β_2 -microglobulin (β_2 -m), the 10.9 kDa light chain of type-I histocompatibility complex, which can seed as amyloid fibrils during long-term hemodialysis treatments. Like PrP, β_2 -m dimerizes through 3D-DS [118] and forms propagated domain-swapped amyloid fibrils stabilized by disulfide bonds [112] (Figure 11 C).

Again, other amyloidogenic proteins dimerize and massively aggregate through 3D-DS. These are: i) the immunoglobulin G-binding B1 domain, which forms 3D-DS conformationally different dimers [119] and tetramers [120] induced by core-domain mutations before forming fibrils [121]; ii) T7-endonuclease I which forms runaway domain-swapped fibrils stabilized by core-domain intermolecular disulfides [122]. iii) Cell cycle protein Cks1, which fibrillize through the preliminary formation of a domain-swapped dimer [123]. Conversely, for another important amyloidogenic protein, TTR, 3D-DS is to date only hypothesized [124], while the direct stacking model interaction between subunits [125] is the one still nowadays accepted.

Before leaving the “3D-DS toward amyloidosis” topic, it has to be mentioned that wt RNase A, despite its high structural versatility [5], and its high SC, and although displaying some amyloid-prone short sequences [126, 127], is not able to form amyloid or amyloid-like (i.e., *in vitro*) fibers. Its core domain, in fact, is stabilized by four disulfide bonds, and ‘self-chaperones’ the whole protein from falling towards fibrillization [128]. Incidentally, the only pancreatic-type RNase known to date to form fibrils is the eosinophil cationic protein ECP [129]. Again, Eisenberg and co-workers showed that, contrary to wt RNase A, some mutants, such as poly Q- and poly G-RNase A, spontaneously form “native-like” amyloid fibrils through C-terminal and N-terminal 3D-DS, respectively [128, 130]. The term “native-like” is referred to the evidence that the core-domains of each protomer forming the fiber remain conserved and natively structured in it [128, 130]. These findings confirm once again that 3D-DS and cross- β -zipper spines are events that can overlap [72].

Finally, the 3D-DS protein dimers or oligomers mentioned above are homo-polymers, and almost all domain-swapped proteins nowadays known are indeed homo-oligomers. Domain-swapped hetero-oligomers are extremely rare, but one to be mentioned is the IX/X-bp anticoagulant complex. This is a domain-swapped dimer forming between two homologous subunits which cross-associate through an intermolecular disulfide bond, but also intertwining a flexible loop located in the central part of each subunit [131]. Contrary to what it could be expected, the dimeric hybrid obtained by associating RNase A with a monomerized BS-RNase [85] did not show a domain-swapped nature. It consists, instead, of two different conformers associated through hydrophobic and electrostatic interactions [132].

4. Stability of the protein oligomers

Protein oligomers are supramolecular structures which are sometimes 'chosen' by nature *ab-initio*, or often built up as a response to natural or non-natural events. Anyway, in both cases oligomers can follow different fates. They can be highly stable, mainly when formed by irreversible phenomena, or can represent metastable or even transient events, thus undergoing fast or slow dissociation. Therefore, there are clearly several cases to be analyzed.

First of all, covalently linked oligomers are obviously the most stable supra-molecular protein structures, except if they interact with proteases, or of course if they have disulfide bonds. Disulfides can be affected by slight redox changes of the environment and reduced to free cysteines or cysteine-like adducts, thus unchaining the two (or more) covalently linked protein molecules. This can be the case of hetero-dimers formed by immunotoxins or other artificial conjugates that can be released in the cell by the reducing power of cytosol [21]. A similar but natural event is what concerns BS-RNase, whose two intermolecular disulfide bridges can be reduced in the cytosol with the formation of two monomers derived from the M=M isoform, while a non-covalent dimer (NCD) [133] survives from MxM [55] thanks to the 3D-DS of its N-termini.

The majority of protein oligomers forms through non-covalent weak associations which can often lead to metastable dimers or oligomers. Their lability is essentially related to the nature of the interaction(s) between the subunits, and to the extent of interface area. H-bonds are weaker than electrostatic interactions, but they can be crucial in anchoring a domain into a specific orientation that can be further stabilized by hydrophobic or electrostatic interactions. Thus, the balance of enthalpy and entropy contributions is decisive in driving a protein oligomer to survive or not, taking into account that entropy is against protein-protein association, a phenomenon which is instead favored by a high concentration of the protomers [37, 50, 77], as was reported above in equations 1 and 2.

For example, human AGT (see Figure 6 a) is active only in the dimeric status, while the enzyme loses its activity if dimerization is hindered. This occurs because the two composite active sites are incomplete despite a PLP co-enzyme is present in each subunit [41]. It is now well known that mutations destabilizing the large interface located between the two subunits can dramatically lower the activity of AGT and possibly drive the enzyme towards monomerization [35]. This latter event induces an unwanted and uncontrolled aggregation which traps monomers, blocking the natural dimerization of AGT [35]. The pathologic effects due to the loss of dimeric, or in general oligomeric, native structures is a general feature of PLP-dependent enzymes.

In general, mutations can affect the overall conformation of protein dimers and/or oligomers and induce monomerization or vice versa uncontrolled aggregation, and even fibrillization. This is the case of TTR (see above), whose native homotetramers are destabilized by point mutations, such as V30M or L55P. The tetrameric assembly in these variants is weakened and the protein easily monomerizes, then undergoing fibrillization through dimeric and octameric annular intermediates [10]. Another protein whose oligomeric status is dramatically affected

by point mutations is hemoglobin, which extensively self-associates in its sickle-cell variant (HbS).

Formation of protein oligomers can be induced in some cases by point mutations, as is for hp-RNase [91], or as a consequence of changes in environmental conditions (pH, temperature, protein concentration). This is true for the various domain-swapped dimers and oligomers of RNase A and BS-RNase. Indeed, several studies have been performed on these two pancreatic-type enzymes, who display 82% identity [53]. Thus, the 23 out of 124 different residues have been considered as key stabilizing or destabilizing determinants. In particular, in both variants several hinge loops residues and/or others belonging to the swappable N-terminal or C-terminal domains or interacting with them have been mutated [64, 65, 134, 135]. Many of them have been found to be key residues in stabilizing the domain-swapped oligomers, while other were found to promote oligomers dissociation towards the native proteins, i.e., monomeric RNase A or dimeric BS-RNase [134].

Other factors which affect the stability of the oligomers are pH, temperature, ionic strength of the medium. It is easily understandable that acidic or basic pHs destabilize oligomeric assemblies, as well as native monomeric proteins. The role of temperature is very important in stabilizing or destabilizing non-covalent protein oligomers. In fact the subunit motions are proportional to temperature increase, thus, a metastable adduct can be forced to quickly dissociate by increasing the temperature. Furthermore, heat allows a protein to access its denatured state [136] which is a destabilizing event 'per se'. Anyway, a very recent study reveals that low temperatures, beyond affecting the folding of native monomers [137] can be useful to study the denaturation and dissociation of dimers through NMR procedures [138].

All these effects, as well as the role played by ionic strength, are qualitatively and quantitatively different from one case to another, because each oligomeric complex can display different shapes, dynamics, and intersubunits surfaces.

In addition, dynamic motions can differently affect the stability of oligomeric conformers belonging to the same protein. This is the case for RNase A whose dimers display a different flexibility, higher for C_D than for N_D [139, 140]. The dynamics of multimeric assemblies dissociation can lead towards different pathways, then producing different smaller products. This is true, for example, for porphobilinogen synthase (PBGs), an octamer which can dissociate to tetramers and dimers either symmetrically, through a consecutive loss of dimeric adducts, or through an asymmetric detach of one subunit per time [141].

The concentration of a protein is crucial either to oligomerize or dissociate, or even evolve toward larger multimers and fibrils. These phenomena are ruled by the K_D values associated to each oligomerization process (see equations 1 and 2 and Figure 4), thus dilution can be a mean by which oligomers and multimers can be destabilized and dissociated [93]. Consequently, macromolecular crowding in general [142] can deeply affect the propensity of a protein to oligomerize by influencing the oligomerization yields [143] and/or the dissociation pathways and kinetics [74].

Also proteolysis, occurring naturally or induced, limited or massive, is a way through which proteins' activities are naturally or artificially switched on/off, or anyhow tuned. Thus, oligomerization can also affect proteolysis: in fact, a domain that in a native monomer is exposed to the action of a proteolytic agent can be partially or totally hidden by dimerization or oligomerization, and proteolysis can be slowed down or even blocked; otherwise, a region which in the native monomer is well structured and therefore protected, or even hidden, can be destabilized, destructured and exposed after oligomerization, becoming susceptible to proteolysis. This is what happens with RNase A, whose N_D is definitely more susceptible to proteolysis than the C_D conformer [144].

Another interesting case is represented by the pore forming toxin (PFT) families, which are most often produced as soluble monomers, proteolitically cleaved by host proteases leading to their oligomerization and pore formation. This occurs, for example, with aerolysin [145], with protective antigen (PA) from anthrax [146], and with thiol-activated cytolysin (TACY) pore forming family [147].

Finally, it has to be underlined that all factors able to stabilize/destabilize protein oligomers, i.e., pH, temperature, ionic strength, protein concentration, molecular crowding, mutations, are not independent of each other. This makes the scenario more complicated than expected and clearly indicates that an 'absolute stability' of a protein can not be easily defined. This is true, for example, for RNase A domain-swapped oligomers (see Figure 9). Their high number of conformers and the possible interconversions between oligomers make the picture quite complicated [74], and only some data concerning dimers' stabilities have been reported to date [89, 148, 149]. With an apparent contradiction, RNase A- N_D was sometimes reported to be more stable than C_D while in other environmental conditions the situation is the opposite. These data clearly indicate that different combinations of all the environmental conditions reported above are crucial to stabilize or destabilize different dimeric or oligomeric structures.

5. Oligomeric proteins: functional vs aberrant interactions

5.1. Gain or loss of function(s) after protein oligomerization

Natural oligomerization of proteins has been settled by evolution in order to obtain a control of their biological features, for instance of enzyme activity. In some cases, proteins are inactive unless they dimerize or oligomerize because of the high hydrophobic surface the monomer exposes to the solvent, as reported above for PLP-enzymes. Oligomerization can be constitutive, such as for PLP-enzymes, or induced by signal molecules, like for membrane proteins. Self- or hetero-association of proteins can also be artificial, but can in any case lead to new activities or block activities that in certain situations can become unwanted, for example in the feedback control of some enzymes.

Dimerization and oligomerization can also increase or lower pre-existing activities. In this case the phenomenon can be considered as a sharp controller and tuner of important

activities. These gain- or loss-of-function events occur by exposing or hiding active surfaces or, for example, by inducing positive or negative allostery impossible to be warranted by the native monomer. These events can be ruled not only by changing the oligomerization/polymerization status of the proteins, but also by conformational changes induced by ligands, such as it occurs with Hb (see Figure 5). Gain or loss of native functions induced by oligomerization can be benign or malignant and also nature can drive either towards physiologic or pathological events, as it happens, respectively, with actin [150] or with sickle-cell anaemia associated to a point mutation in hemoglobin (HbS). Other important pathological implications, i.e., oligomers evolving towards fibrils, will be discussed later. A benign case that deserves to be mentioned is rhodopsin, the pigment involved in the phototransduction events that are crucial for vision, which physiologically organizes itself in a supra-molecular ensemble to be active [151].

An interesting case in which protein oligomerization induces a gain of function concerns RNase A. The native monomeric enzyme only degrades single-stranded (ss) RNA and is not cytotoxic, while its artificial dimers and oligomers, either forming through non-covalent 3D-DS or covalent bonds become also active against double-stranded (ds) RNA [29, 57, 73, 152]. Moreover, they can become selectively cytotoxic towards cancer cells both *in vitro* and *in vivo* [153-156], although more recent results indicate that cytotoxicity can be dependent also on the type of cell line studied [32]. This acquired cytotoxic power can be considered benign, given the selectivity towards malignant cells, and can be mainly ascribable to the possibility of oligomeric RNases to evade the ribonuclease inhibitor (RI), which is designed for tightly trap monomeric RNases [157]. This is also the main reason why only MxM BS-RNase (see Figure 8) is cytotoxic: in fact, the cytosolic reducing environment allows only the MxM domain-swapped isoform to survive as a non-covalent dimer (NCD) and to evade RI, while M=M becomes a monomeric, RI-susceptible, derivative [158]. Another example of cytotoxic protein oligomers is represented by the ones formed by p13suc1 [159]. The native cell-cycle regulatory protein is a monomer/DS-dimer mixture [160] that can form large native-like cytotoxic aggregates through 3D-DS, and whose structural determinants governing the swapping mechanism have been extensively studied as reported above [62, 68]. Thus, the artificial induction, quench, or control of the oligomerization event(s) can be useful to avoid, induce, or tune several biological properties of proteins, such as for example enzymatic activity, or the incoming of cytotoxicity.

5.2. Protein oligomerization towards fibrillization and/or amyloidosis

Protein self- or cross-association can be naturally or artificially controlled to the degree of oligomers or multimers, but can sometimes undergo uncontrolled massive aggregation, often resulting in fibers, as it has already been reported in the domain swapping section. These supra-molecular structures can be benign, such as in muscle tissues (actin [150]), or very often harmful. The latter case is true for sickle cell HbS insoluble fibrous malignant polymers, or for amyloid or amyloid-like fibrils, which are often, although not always [2, 15], associated with deleterious neurodegenerative diseases [37]. Proteins can become prone to fibrillization

because of ageing, or after changes in the environmental conditions, such as crowding, pH or temperature shocks. In addition, also point mutations can induce and often speed up the fibrillization phenomena, as for TTR V30M or L55P [161, 162], or for the human prion protein [163], or also for the homo-tetrameric p53 tumor suppressor protein [164, 165]. Several lethal amyloidoses, for example linked to TTR or β_2 -m, or to other toxic fibrillogenic proteins, like prions, display a premature incoming when associated to familial pathogenic mutations or overpass the species transmission barriers [166]. Then, also cancer has been recently considered a possible prion-like disease, due to the fibrillogenic behavior of some p53 mutants [167].

In this complex scenario, it is known [163] and is more and more accepted that the first oligomeric/multimeric species preceding the formation of protofibrils and fibrils are the toxic agents responsible for the incoming of the associated pathology(ies) [2]. Thus, a lot of work is presently devoted to discover the structural and functional properties of these 'toxic oligomers', which is a very difficult endeavor indeed, because these species show a great tendency to fibrillize quickly.

In the last five years, the evidence became clear that oligomers produced by the same protein can be toxic or non toxic depending on the way they are produced [2, 7, 15]. This was found, for example, with the *Escherichia Coli* hyperforin-N (HypF-N) spherical oligomers [15] and also with other amyloidogenic proteins. Several different supramolecular large structures can be detected with new techniques, such as solid-state NMR, Cryo-TEM, High-Resolution Atomic-Force Spectroscopy, Molecular Modeling. For example, TTR has been discovered to fibrillize through the formation of annular oligomers deriving from the monomer which had been in turn detached from the native tetramer [10].

Several other amyloidogenic proteins have been extensively studied. Among them, the number of those that follow the 3D-DS mechanism is continuously increasing [80], such as for hPrP, hCC, β_2 -m, the properties of which have already been discussed. In the last years several proteins initially not considered to follow 3D-DS were discovered instead to undergo this mechanism. Nevertheless, the mechanism by which an oligomer/fiber may form essentially depends on the symmetry of the interfacial association and 3D-DS can be not mandatory to reach this requisite. Thus, the formation of the cross- β -spine fibrils intima could follow an end-to-end stacking mechanism, the same followed by non-amyloidogenic proteins, such as hemoglobin-S [164], or by non-harmful proteins, such as actin [170] or tubulin [171]. Anyway, considering that amyloid fibers are not oligomers, several oligomeric precursors of amyloidogenic proteins are continuously studied, despite their transient nature. Among them, human lysozyme, *Sulfolobus Solfataricus* acylphosphatase (AcP), human superoxide dismutase-1 (SOD1), the latter associated with the devastating disease amyotrophic lateral sclerosis, have been deeply investigated in their propensity to undergo amyloid fibers through amyloidogenic oligomers [167]. Further detailed studies, which are beyond the topic of this chapter and focused on the nature of amyloidogenic oligomers and their differences from the non-amyloidogenic ones have been recently reviewed [2]. Several interesting news concerning the oligomers' polymorphism and the structural rearrange-

ments they can undergo (Figure 12) to follow a harmful fibrillogenic pathway or a non fibrillogenic harmless destiny [7] can be found in that review [2].

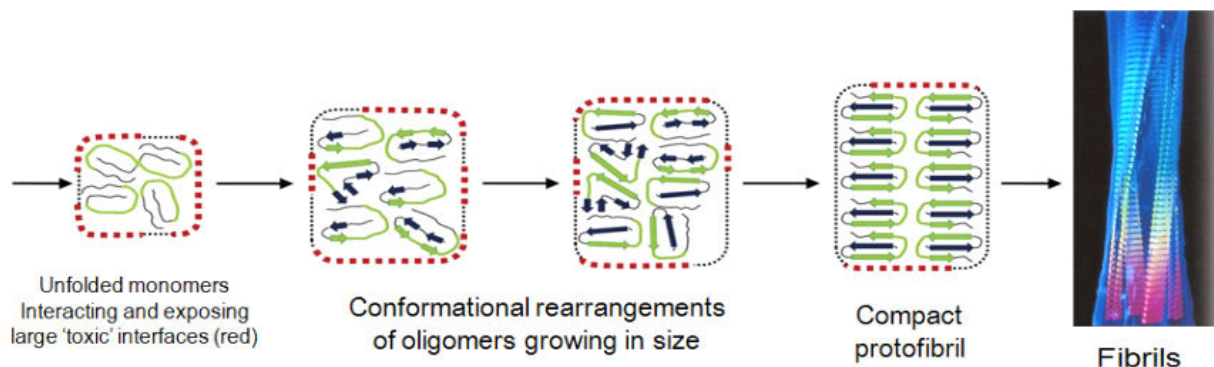


Figure 12. Evolution of partially or totally unfolded protein monomers towards ordered fibrils through oligomeric intermediates displaying different sizes and conformations. (Modified from [2] and [88]).

6. Oligomeric proteins and industry

Besides artificial chemistry products, industry has often used natural or artificially modified bio-products to produce bio-materials or bio-fibers. In this context, nano-particles and nano-materials certainly represent a new fascinating frontier to be developed.

Peptides and proteins can be driven towards controlled oligomerization, polymerization and/or fibrillization to form products displaying useful physico-chemical and mechanical features to be adopted for new or renewed industrial applications. Incidentally, several efforts have been performed in the engineering of short peptides and small biomolecules [168-170], while definitely less is known on the industrial application of protein oligomers, multimers and/or fibrils. The most common materials formed from peptides and proteins are hydrogels, which can be applied in tissue engineering and in drug delivery [169]. These materials are typically formed by hydrated cross-linked fibers that somehow resemble the agarose or polyacrylamide gels extensively used in biochemical laboratories.

The 'intima' of these hydrogel structures and their morphology define size, biocompatibility, and mechanical, elastic or rigid properties. Anyway, only some hints concerning the interesting 'industrial' applications of protein oligomers will be given here.

For example, the 3D-DS mechanism has been reported as to be applicable in material design [94]. Indeed, domain-swapped oligomeric peptides have been produced to obtain hydrogels [171], while also proteins can be artificially designed to undergo 3D-DS to form oligomers which could be useful to produce biomaterials [172]. Beyond small oligomers, the ability of some proteins to become fibrillogenic through propagative or runaway domain swapping [80, 94] could, or should, be exploited in order to engineer variants that could form harmless 3D-

DS fibers with special morphological and mechanical properties. These supra-molecular structures could also become materials devoid of direct biological applications, but with industrial and ecologic relevance. Indeed, proteins that for example combine 3D-DS with a covalent stabilization of their fibrillar products through the formation of novel disulfides (like β 2-m and recombinant PrP) [110, 112], should be useful to obtain reversible products that could be easily unstructured and recycled under reducing conditions, and, thus, without negative ecologic consequences.

Many examples of protein 'benign' (non-amyloidogenic) fibers could become precious for industry applications. An example can be represented by the trimeric hexon protein, displaying a novel triple β -spiral fibrous fold with implications for the design of a new class of artificial, silk-like fibrous materials [173].

Anyway, all the potential industrial applications of protein oligomers, multimers, and fibrils are certainly far to be completely explored, and what we have here reported about 3D-DS applications confirms that further deep investigations deserve to be performed.

7. Conclusions

All the notions reported in this chapter indicate that the complexity of the protein oligomerization topic augments every day. In particular, the increasing number of studies focused on natural protein oligomerization and the improvement of the quality of the investigation techniques have greatly enlarged the complexity of the analysis of the structural and functional features of protein oligomers. Furthermore, the sharpening of the strategies used by chemistry to obtain cross-linked artificial oligomers allowed industry and laboratories to obtain less heterogeneous products, which were, in addition, scarcely modified with respect to the native monomeric protein(s).

Again, the discovery that some proteins can naturally oligomerize by combining covalent linkings with weak interactions made the scenario even more complicated. This is the case, for example, of proteins that form domain-swapped fibrils additionally stabilized by newly forming disulfides. The same is true also for membrane proteins that associate and sometimes covalently stabilize their interaction as a response to effectors which can play the role of activators or quench cell signals.

Thus, the aim of this chapter is to focus the attention of the reader on the principal features of protein oligomerization. We kept separated, when possible, the covalent linking oligomers from the non-covalent protein self-association products, as well as the natural, constitutive or induced, events from the artificial ones. When we were not able to separate these aspects well, we tried to give a picture as clear as possible. On the other hand, this underlines how much increasing interest has and how many further studies deserves the topic of protein oligomerization.

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