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Review

Biological activity and pathological implications of misfolded proteins

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Abstract. The physiological metabolism of proteins guarantees that different cellular compartments contain the appropriate concentration of proteins to perform their biological functions and, after a variable period of wear and tear, mediates their natural catabolism. The equilibrium between protein synthesis and catabolism ensures an effective turnover, but hereditary or acquired abnormalities of protein structure can provoke a premature loss of biological function, an accelerated catabolism and diseases caused by the loss of an irreplaceable function. In certain proteins, abnormal structure and metabolism are associated with a strong tendency to self-aggregation into a polymeric fibrillar structure, and in these cases the disease is not principally caused by the loss of an irreplaceable function but by the action of this new biological entity. Amyloid fibrils are an apparently inert, insoluble, mainly extracellular protein polymer that kills the cell without tissue necrosis but by activation of the apoptotic mechanism. We analyzed the data reported so far on the structural and functional properties of four prototypic proteins with well-known biological functions (lysozyme, transthyretin, β2-microglobulin and apolipoprotein AI) that are able to create amyloid fibrils under certain conditions, with the perspective of evaluating whether the achievement of biological function favors or inhibits the process of fibril formation. Furthermore, studying the biological functions carried out by amyloid fibrils reveals new types of protein-protein interactions in the transmission of messages to cells and may provide new ideas for effective therapeutic strategies.

Key words. Amyloidosis; protein misfolding; lysozyme; β2-microglobulin; apolipoprotein AI; transthyretin; β protein.

Introduction

One of the most intriguing and fascinating phenomena of life is the structure-function relationship of proteins. A still largely obscure code, intrinsically present in the primary structure of proteins, drives the formation of a superior three-dimensional (3D) structure [1]. This entropically negative event is regulated by sophisticated thermodynamic parameters that determine the folding kinetic, the folding stability and much of the protein metabolism. In the last decade better knowledge of protein-folding pathways followed the observation that several human diseases are caused by abnormalities of protein folding and metabolism, not simply diseases due to the loss of an irreplaceable function of an incorrectly folded protein but also diseases in which proteins lose their natural architecture and self-aggregate in a toxic fibrillar structure denominated amyloid fibril. This im-

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plies that for some polypeptide chains there is a pathological folding pathway alternative to physiological and productive ones. Extensive efforts have been made in recent years in an attempt to define the molecular basis of the 'chameleon' behavior of these proteins [2]. More is known about the mechanism of amyloid fibril formation, and the finding that the origin of this pathological phenomenon arises from abnormal kinetic and thermodynamic behavior of an intermediate of the folding pathway is also more evident [3].

The protein that makes fibrils derives from a precursor that is synthesized in a biologically active native state and the conversion of the native protein into the aggregate state is associated with the loss of its physiological function. The abrogation of biological activity is not simply due to solubility changes or caused by the new environment in which the protein is located, that is from biological fluids to connective tissue, but is also the result of relevant conformational modifications affecting the tertiary and quaternary structure and perturbation of the protein active site. This fibrillar material, on the other hand, cannot be considered biologically inert; fibrils interact with complex carbohydrates that bind fibril both during the fibril formation process as well as in the final structured state, and fibrils bind the so-called accessory proteins like serum amyloid P component (SAP), apolipoproteins and several protease inhibitors that colocalize the fibrillar deposit. Furthermore, the fibrils interact with cells and apparently exert a toxic effect on cell life not only through physical displacement of normal tissue by the growing proteinaceous mass but also by peculiar fibril-membrane interactions that end with the commitment of the cell to the apoptotic pathway. It is interesting to note that the acquisition of these biological functions plays a role in protecting the fibrils from reabsorption and in favoring their deposition, growth and substitution for the normal tissue architecture.

We will now review the structural and thermodynamic data regarding the state of folding in which biological function is achieved in four proteins (lysozyme, transthyretin, β2-microglobulin and apolipoprotein AI) that play sophisticated roles in the correctly folded state but can become a cause of amyloid disease in the fibrillar aggregate, and we will focus our attention on the influence that achievement of biological function has on fibril formation.

Lysozyme

This is a 14.7 kDa protein whose function in humans is probably not completely understood, but which is known to have a protective effect against bacterial infection through its capacity to hydrolyze the bacterial wall. Lysozyme catalyzes the hydrolysis of natural and synthetic polymers of $\beta(1-4)$-linked units of N-acetylglucosamine (NAG) and N-acetylmuramic acid. The substrate binds the enzyme in a well-defined deep cleft located on one side of the molecule. The discovery of two cases of familial amyloidosis caused by lysozyme variants [4] represented a unique opportunity for studying the structure-function relationship in amyloidogenic proteins; in fact, human lysozyme is one of the best-studied enzymes in terms of catalytic properties, structure and folding dynamics. The two lysozyme species able to form amyloid fibrils in vivo have single amino acid mutations, either threonine for isoleucine at position 56 or histidine for aspartate at position 67. Both substitutions occur at highly conserved positions in the sequence and do not involve amino acids directly implicated in the catalytic reactions. Both variants are less thermostable than the wild-type protein, with a midpoint of denaturation 12 °C lower at pH 5 [5]. The conformational dynamics of the two variants was studied by monitoring the hydrogen-exchange labelling by electron spray mass spectrometry; very little protection from exchange was shown in both amyloidogenic lysozymes. This finding has suggested that the cooperativity of the native fold is reduced, and the frequency of the native state fluctuations is increased to an extent that permits the access of water to the interior of the protein [5].

Both variants can fold in a nativelike state and display glycolytic activity against the natural substrate (the bacterial wall) and hydrolyze the synthetic polysaccharide chitopentoside. The kinetic properties of the variants have shown normal enzymatic parameters in the Thr56 variant and abnormal k_m and k_{cat} for the His67 variant with respect to the wild-type. In the latter variant this finding fits well with a distorsion of the catalytic cleft of the enzyme demonstrated in the 3D structure (fig. 1). The mutations of both variants alter the stability of the nativelike state which, near the melting point at physiological pH, highly populate a state of partial unfolding (molten globule) that is unknown at the same pH for the wild-type species. Structural conversion into a fibrillar aggregate was carefully monitored by infrared spectroscopy and appeared to pass through the loss of the prominent α structure, an increase of the β sheet and complete loss of enzymatic activity. The tertiary structure of the single lysozyme molecule and its spatial orientation inside the natural fibril is not known, but the primary structure is not modified by proteolysis, glycation or other processes except Met oxidation. Fibril solubilization by guanidine reverts the lysozyme structure to a random coil state and opens the molecule to the refolding pathway that ends in opportune pH, with the creation of a functionally active enzyme. The antithesis between the correctly folded state (biologi-

cally active) and the fibrillar or misassembled state (biologically inactive) suggests that any procedure favoring the former condition could have therapeutic implications. The binding site of lysozyme could represent a therapeutic target because the capacity of the N-acetylglucosamine trimer $(NAG)_3$, at millimolar concentrations, to stabilize the native conformation is known.

It is probably worth summarizing some of the data available on the structure and behavior of the catalytic site. The side chains of polar residues that create the binding site are hydrogen-bonded to the acylamino and hydroxyl groups of the substrate, and the apolar residues make contact with the hydrophobic region of the substrate. The cleft is divided into six sites—A, B, C, D, E, F—and the bond that is cleaved lies between sites D and E in proximity of Glu35 and Asp52. The reaction proceeds through a carbonium ion intermediate which is stabilized by the carboxylate of Asp52. The trisaccharide used as a competitive inhibitor in crystallization experiments [6] or the fluorescent disaccharide used in the investigation of the lysozyme folding kinetics [7] are low-affinity inhibitors (K_d in the range of 1×10^{-5} M) while on the other hand it is known that a transition state analogue in which the lacton ring mimics the carbonium ion-like transition state tightly binds lysozyme with a K_d of 8.3×10^{-8} M [8]. The studies performed on the folding dynamics and stability in the presence of lysozyme competitive inhibitor suggest that the ligand has no influence on refolding kinetics, because the creation of the active site is achieved after the formation of the β domain and its integration into the native structure. By contrast, it has been shown that competitive inhibitors have a stabilizing effect on the native state; in fact, the unfolding rate decreases sixfold in the presence of 8 mM $(NAG)_3$, and the melting temperature increases by about 6 °C [9]. The effect of the inhibitor in locking the lysozyme native structure was also confirmed by nuclear magnetic resonance (NMR) studies of the complex inhibitor/egg-white lysozyme [10]. A stable ligand inhibitor with a greater affinity than that of $(NAG)_3$ would be desirable, especially because at least in one of the two amyloidogenic variants structural and enzymatic kinetic data have shown reduced affinity for the substrate, and therefore any therapeutic attempt would require giving the patient huge amounts of such a compound.

Another aspect of lysozyme biology that is most likely relevant in these cases of familial amyloidosis is tissue distribution and interaction with other proteins. Lysozyme synthesis occurs in all human tissues, and the protein is present in all biological fluids from tears to plasma where it is mainly sustained by neutrophils, leukocytes and monocytes [11]. Lysozyme is rapidly removed from plasma by kidney filtration, and it has been calculated that in 1 h approximately 76% of plasmatic lysozyme is excreted in the urine. A careful analysis of data regarding lysozyme distribution suggests that

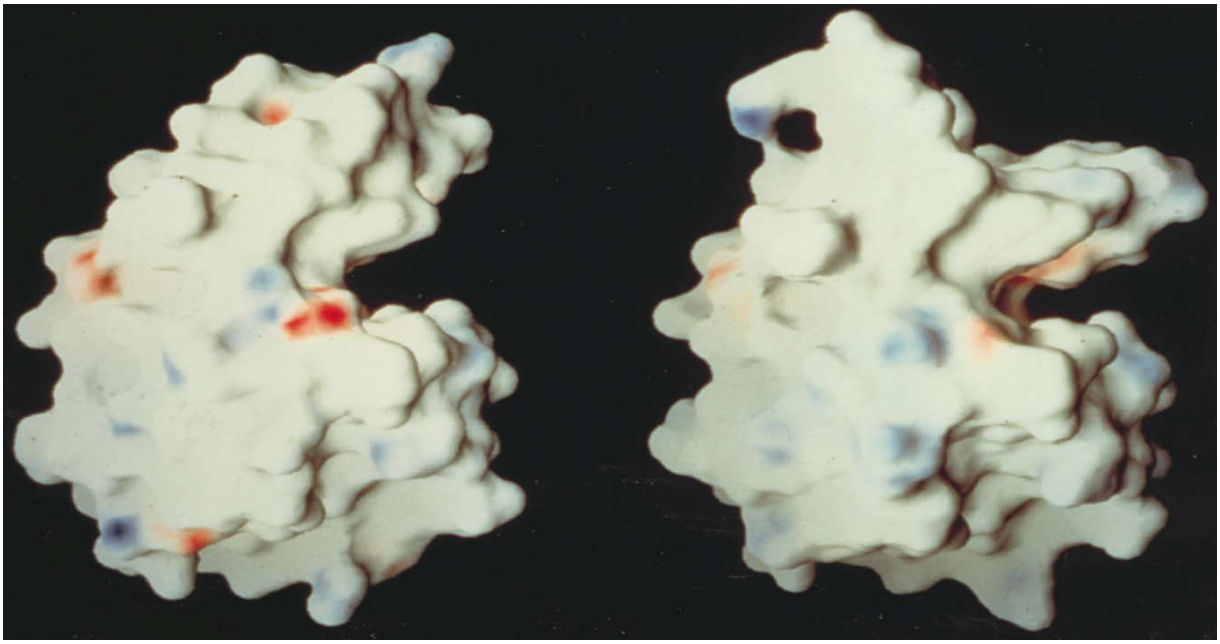

Figure 1. The molecular surface of wild-type human lysozyme (left) and amyloidogenic Asp67His variant (right). This figure was produced by Margaret Sunde and Colin Blake using the programme GRASP [110] with the coordinates deposited in the Brookhaven PDB 1LZ1 and 1LYY.

there is a certain discrepancy between the synthesis and tissue localization of radiolabelled lysozyme in the liver and spleen; indeed, $\sim 5\%$ of lysozyme is synthesized in the liver and spleen, but the two organs are able to take up more than 25% of the circulating enzyme. This suggests that circulating lysozyme is physiologically taken up by liver and spleen structures, and it is worth noting that these two organs are massively involved in this type of amyloidosis. An interaction between lysozyme and other constituents of the target organs can be hypothesized, and this is not surprising for such an extremely cationic protein. In this regard it is relevant that a strong interaction between lysozyme and elastin has been clearly documented [12], and this finding could offer an explanation for a peculiar pathological presentation described in a young patient with lysozyme amyloidosis whose liver was particularly friable and susceptible to spontaneous hemorrhage. A small amount of deposited amyloid was associated with a striking absence of reticulin staining [13]. It is known that reticulin contains a considerable amount of elastin, so the accumulation of lysozyme on the fibroelastic network of the liver might be driven by a putative target protein such as elastin.

Transthyretin

The structural modifications associated with fibril formation have been extensively studied in transthyretin (TTR), whose structure-function relationship is also well known. Human TTR (in association with thyroxine-binding globulin) and serum albumin distribute thyroid hormones in the intravascular and interstitial compartment and control thyroid hormone metabolism and activity [14]. TTR is also secreted by the choroid plexus toward the brain and represents the main thyroid hormone-binding protein in the cerebrospinal fluid [15]. TTR also transports retinol from the liver to target cells in a complex with retinol-binding protein [16]. The protein has a predominantly β-sheet structure and adopts a tetrameric configuration under normal physiological conditions, as discerned from the 1.8-Å crystal structure determined by Blake et al. [17]. The thyroxine (T4) binding channel in TTR is a central channel lined with four monomers. Each TTR monomer is composed of eight β strands, designated A–H, that are organized in two β sheets, namely DAGH and CBEF. The CBEF sheet is oriented toward the exterior of the molecule, and sheets DAGH line the binding channel, defining two T4 binding sites. The structure of the binding site is well known and defined by hydrophobic and hydrophilic regions in each site that match the structure of T4.

In certain individuals around the age of 80, wild-type TTR is converted into amyloid that causes senile systemic amyloidosis (SSA), a disease sometimes characterized by symptomatic heavy amyloid deposits in the heart. In familial polyneuropathy amyloidosis (FAP), variants of TTR are involved in the amyloid formation, and so far more than 60 different TTR mutations have been reported to be associated with this form of amyloidosis [18].

TTR in the wild-type species forms amyloid in vitro upon partial acid denaturation and during pH-mediated reconstitution from a partially unfolded state [19, 20]. The best-characterized intermediate in the denaturation/reconstitution pathway is a structured monomer that primes the process of unlimited polymerization [21]. This intermediate has substantial β-sheet structure with a nonnative configuration but still displays peculiar tertiary interactions over the pH range 5–3.9. The amyloidogenic intermediate undergoes concentration-dependent assembly into several quaternary structures that lead to TTR amyloid fibril formation at physiological TTR concentrations [22]. Studies of limited proteolysis have shown that the TTR tetramer is resistant to the action of V8 protease at pH 7.5, but is rapidly cleaved at pH 4.4, the value at which the amyloidogenic intermediate is highly populated [23]. Peptide analysis demonstrated that the C-strand-loop-D-strand portion of TTR is sensitive to the action of the protease, suggesting that this portion of the polypeptide chain becomes disordered and moves away from the core of the protein at pH 4.4. This structural rearrangement, which is peculiar to the amyloidogenic monomer intermediate, could be important for facilitating self-assemby into amyloid fibrils. The importance of this structural rearrangement in the aggregation process also stems from the finding that many FAP variants (as well as the wild-type form) isolated from different patients are cleaved in this portion of the TTR molecule [24, 25].

The single-site mutations that are associated with FAP destabilize the tetrameric form of TTR in favor of the monomeric amyloidogenic intermediate [26]. Under mild denaturing conditions at which wild-type TTR is stable, the FAP variants populate the monomeric amyloidogenic intermediate and self-assemble into amyloid. The dynamic interaction of TTR subunits in the wild-type and Val30Met mutant, the commonest amyloidogenic variant, has been studied by a technique that monitors the hydrogen exchange by mass spectrometry [27], and the results have shown reduced protection from hydrogen exchange in the amyloidogenic protein. However, the TTR mutations associated with FAP do not significantly alter the tertiary structure of the native state. The X-ray crystal structure of amyloidogenic Val30Met is very similar to that of wild-type TTR, although slightly different modifications were observed

in the 3D structure of the recombinant Met30 variant resolved by Blake's group, who found a movement of His56 that could modify the entrance of the cavity in which T4 is located, and this could justify the reduction of the apparent association constant (K_a) from 1.39×10^7 M^{-1} found in the wild-type to 0.24×10^7 M^{-1} in the Met30 variant [28]. In the X-ray structure of the same TTR variant, purified from plasma and resolved by Hamilton and co-workers, a decrease in the width of the T4 cavity at the A strand is also evident [29]. These data suggest that the thyroxine binding site is a sensitive indicator of tetramer stability and demonstrate the existence of a certain inverse correlation between amyloidogenicity and affinity for thyroxine. This idea is also supported by the demonstration, in the nonamyloidogenic Thr109 variant, of an opposing modification that induces an enlargement of the cavity and increases the affinity for T4 [30]. Another interesting variant is Thr119Met, which is not only nonamyloidogenic but even protects against the disease in patients who present double heterozygosis for Met30–Met119. This variant displays an affinity for T4 that is twice as great as the wild-type form, and the presence of the Met119 replacement stabilizes the TTR tetramer toward dissociation and is unfavorable to the formation of the amyloidogenic intermediate [31].

In the recently determined 3D structure of Leu55Pro TTR [32], it was demonstrated that the Pro for Leu substitution prevents the formation of hydrogen bonds between strands A and D, making the 3D structure of the TTR subunit very similar to the amyloidogenic monomer intermediate structure previously discussed, and although the tetrameric structure is conserved, assembly results in a perpendicular rather than a parallel orientation of the T4 binding channels that displays an extremely low affinity for T4. The abnormal assembly of the monomers creates a tubular-type aggregate that the authors consider the possible nucleus for fibril growth.

Besides the binding to thyroid hormones, there are two other biological functions of TTR that could be pertinent to its pathological behavior: its binding to retinol-binding protein (RBP) and its interaction with a specific cellular receptor. The interaction with RBP has found to be severely perturbed in only one TTR variant, the Ile84Ser variant [33]. This behavior appeared to be a distinct property of this variant and not a general feature of the amyloidogenic TTR. The relevance of the interaction between TTR and a specific cellular receptor and its implication in the amyloid metabolism and tissue tropism of TTR fibrils will probably be elucidated in the near future when a detailed characterization of the receptor is available. Its existence has been proven in chickens [34] and partially characterized in humans [35]. At the present time, however, the thyroxine binding site appears to represent the most promising target for pharmacological inhibition of the conformational changes responsible for fibril formation. The possibility of improving the stability of the tetramer by filling the binding site with natural or synthetic ligands was extensively explored by Kelly's group. In a first study this group used ultracentrifugation to show that T4 and the synthetic ligand 2,4,6-triiodophenol stabilize the tetramer and at micromolar concentrations inhibit fibrillogenesis [36]. More recently, the same group obtained evidence that flufenamic acid acts as a powerful inhibitor of fibril formation: two molecules of flufenamic acid occupy the T4 binding site with affinities of 30 and 255 nM. Extremely high affinity was also found for the Val30Met and Leu55Pro variants despite the reduced affinity for T4 displayed by this latter variant [37]. Crystallization of the TTR-flufenamic acid complex and resolution of the 3D structure elucidated the molecular basis of the increased stability of the wild-type species tetramer in great detail. The conformational modifications induced by the presence of flufenamic acid include rearrangement of the Ser117 side chains on each monomer, which rotate 120° and establish four new hydrogen bonds per tetramer. A similar effect is displayed by the Thr119 side chain, which creates a new hydrogen bond with a water molecule that in turn is hydrogen-bonded to the carboxyl oxygen of Asp18 on an adjacent subunit of the tetramer [38]. The mechanism of inhibition of fibrillogenesis by natural or synthetic ligands in the case of TTR variants which bind them at the binding site with very low affinity is still under debate. In these cases it can be hypothesized that, as described for other proteins [39], multiple nonspecific low-affinity ($k_d < 1 \times 10^{-6}$ M) binding sites on the protein surface exist for small hydrophobic compounds like the bisarylamine inhibitors that could interfere with the process of self-aggregation.

β2-Microglobulin and dialysis related amyloidosis

β2-Microglobulin (β2-m) constitutes the light chain of the major histocompatibility complex type I (MHCI). In the MHCI complex, β2-m is folded like the CH3 domain of the Fc fragment of immunoglobulins with β strands that binds the heavy chain through multiple hydrogen bonds. In particular, 595 A^2 of solvent-accessible surface area are buried in the interface with the α3 domain of MHCI. The interface is created by the four-stranded β sheets of β2-m and α3, with the direction of the strands in β2-m being approximately perpendicular to those in α3. β2-m is not involved in any direct contact with the antigenic peptide, but it stabilizes the complex associated with the peptide during the correct folding and assembly of the subunits. Peptide and β2-m

bind cooperatively to the heavy chain [40], and the in vivo assembly of the heavy chain into complexes with peptide and β2-m occurs in the Golgi apparatus [41], followed by final localization of the complex at the extracellular site of the membrane. The physiological process of MHCI catabolism is associated with shedding of β2-m from the complex, transitory persistence of free heavy chain on the cell membrane and, finally, internalization of the heavy chain in the cytoplasm followed by a lysosomal degradative process [42]. However, it has been reported that β2-m-free heavy chain can also be released into the extracellular space from activated cells or leukemia cells through proteolytic cleavage mediated by a metalloprotease at a site close to the membrane [43]. In physiological conditions β2-m is catabolized and excreted by the kidney, but in hemodialysed patients this low molecular weight protein accumulates in the plasma, undergoes extracellular amyloid deposition and causes dialysis-related amyloidosis, a type of amyloidosis in which the fibrils have a selective tropism for the skeletal muscle system [44].

The abnormal protein metabolism of β2-m in hemodialysis patients represents a very complex prototype of disease associated with protein misfolding and misassembly. Hemodialysis is in fact associated not only with a failure of the mechanism of β2-m catabolism but also with an accelerated rate of β2-m shedding from the membrane. Considerable literature exists regarding the impact of different hemodialytic approaches on β2-m metabolism. There is a general agreement that no dialysis procedure can be considered free from the amyloid complication; however, there are numerous, perhaps not conclusive but strongly supportive indications that dialytic procedures performed with more biocompatible membranes could significantly delay amyloid formation [45]. The concept of biocompatibility as referred to the hemodialysis procedure can be defined as the biological consequences of the sum of specific interactions between the blood and the artificial material of the hemodialysis circuit, which generally consist of an inflammatory-like reaction [46] that includes activation of the complement cascade, activation of several proteases, massive redistribution of leukocytes and oxygen radical release. The effect of the hemodialytic procedure on MHCI synthesis and breakdown probably represents key momentum in the regulation of the β2-m supply. Patients on hemodialysis show a slight increase in β2-m synthesis, and it has been reported that cellulose membranes can induce β2-m release from the MHCI located on the membrane of circulating mononuclear cells [47]. A high turnover of the MHCI complex and destabilization of its quaternary structure are of course two unfavorable phenomena in this type of amyloidosis. A detailed characterization of MHCI thermodynamic parameters has demonstrated that filling the binding site with specific peptides can increase the T_m by as much as 26 °C, and the presence of peptide can prevent the release of β2-m from the heavy chain [48]. This represents a very significant example of the strong stabilizing effect played by specific ligands on protein folding. An investigation of the early events in the process of MHCI catabolism and β2-m shedding was recently undertaken by our group, exploiting the properties of a monoclonal antibody that specifically recognizes the C-terminal octapeptide of β2-m [49]. This antibody, named mAb 14H3, was recently described for its capacity to inhibit the process of β2-m fibrillogenesis and was considered to be unable to recognize the MHCI complex on the basis of data obtained using flow cytometry on different cell types. Nevertheless, when we tested this mAb against the soluble form of MHCI removed from the membrane environment, we demonstrated that this antibody can bind the complex with an affinity only one order of magnitude lower than that displayed toward monomeric β2-m. The protection from antibody recognition detected at cytofluorometric analysis appeared to be caused more by the interaction of the MHCI complex with the membrane than by the burying effect of the epitope in the MHCI quaternary structure [unpublished observations]. However, in certain conditions this protection is apparently reduced, and the antibody acquires the ability to recognize its epitope in the MHCI still anchored to the membrane. All the conditions that make such recognition possible are associated with toxic cellular injuries like heat shock, γ irradiation, pH reduction or metabolic stress. Cells that display positive immunostaining of the membrane present DNA fragmentation typical of the apoptotic pathway (fig. 2).

A model of the possible dynamic interaction between MHCI and the cell membrane is presented in figure 3. The condition illustrated in panel A is consistent with a buried epitope, but movement of the extracellular portion of the MHCI is required for antibody access. Reduced adhesion of the complex to the membrane (fig. 3*B*) would provide easier access to a putative protease whose capacity to cut the heavy chain at the membrane site had been previously demonstrated [43], and this appears particularly likely in a condition of protease activation such as that documented during the hemodialytic procedure [50]. Therefore, conformational modifications associated with cell injury can be responsible for increased shedding of β2-m from the cell membrane through a limited proteolytic event, with consequent expansion of the circulating β2-m pool. We demonstrated that the binding of a specific peptide to the MHCI molecule anchored to the cell membrane has the capacity to partially limit the exposure of the mAb 14H3 epitope. The effect of the peptide in packing the core of the soluble MHCI complex was mentioned above [48], but the effect of the peptide on immunolog-

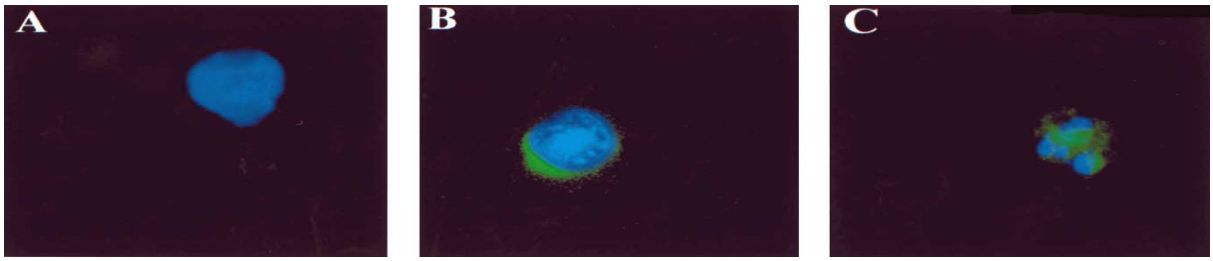

Figure 2. Immunofluorescence analysis of irradiated Jurkat cells stained with the blue DNA marker (diaminophenylindole) and the purified murine mAb14H3 revealed with a fluoresceinated-conjugated goat anti-mouse (green fluorescence). Panel *A* shows a cell with no evidence of apoptosis (14H3-negative); panel *B* an apoptotic cell presenting condensed chromatin in the nucleus (14H3-positive); panel *C* an apoptotic cell presenting a totally fragmented nucleus (14H3-positive).

ical recognition of mAb 14H3 suggests that conformational modifications associated with the formation of the MHCI-peptide complex [51] could influence the strength of the MHCI-membrane interaction.

This model indicates that the phenomenon of fibril formation originates from a process of misassembly between the protein precursor and macromolecules constitutively associated with this protein in the achievement of its function: in this case, the plasma membrane. This model is in some ways reminiscent of the situation of the β-protein precursor, a membrane protein that can cause of Alzheimer's disease (AD) when it is proteolized in a particular way at a site near the plasma membrane. To the best of our knowledge, the role played by the membrane in the modulation of this proteolytic activity that regulates the release of the β peptide has hardly been investigated, but data reported by Racchi et al. [52] imply that changes in the cholesterol content of the membrane can alter its susceptibility to proteolysis.

Going back to β2-m metabolism, once the MHCI complex is shed from the membrane, tissue deposition of circulating β2-m seems to be guided by the affinity of β2-m for collagen. Indeed, amyloid formation occurs mainly at the level of tendons, synovia and bones. The interaction of collagen with β2-m is well established [53] but has not been completely characterized in terms of affinity constant, structure of the binding site and contribution of β2-m to collagen metabolism.

A certain number of biological functions, other than the role played in the MHCI complex, have been demonstrated for monomeric β2-m that can in many ways be related to the pathological phenotype of dialysis related amyloidosis and collagen metabolism. This 99-residue polypeptide at a concentration of 0.3–30 μg/ml (concentration in hemodialyzed patients = 40 μg/ml) is able to induce collagenase synthesis in human fibroblasts [54] and act as a growth factor on calvarial cell cultures [55, 56]. It is not known whether fibrillar β2-m maintains the biological function of the soluble species; however, bone resorption, which can provoke dramatic bone lesions like the ones presented in figure 4, suggests a pathogenic interaction between the protein and bone cells. β2-m tissue tropism is most consistent and predictable in the category of systemic amyloidosis. One reason why fibrillar β2-m retains certain biological properties of the monomeric protein could be the result of overall conservation of the β2-m 3D structure inside the fibrils. The transition from the globular state circulating in the plasma to the fibrillar aggregate deposited over collagen is probably associated in this case with limited conformational modifications. In all the in vitro fibrillogenic methods proposed so far, fibril formation has been obtained in paraphysiological conditions at high protein concentration and low ionic strength [57], in cultures in the presence of cells from patients undergoing hemodialysis [58] or by simple incubation at 37 °C [59]. Furthermore β2-m refolding from ex vivo amyloid fibrils has led to the demonstration, as reported for ex vivo lysozyme fibrils, that β2-m is able to recover a nativelike tertiary structure [60].

The refolding procedure used in the case of β2-m fibrils revealed that the disulfide bridge Cys 25–Cys 80 is conserved in fibrillar β2-m; this covalent bond plays a key role in the tertiary structure pattern [61], and its conservation should restrict the extent of the tertiary modifications that most likely occur in the fibrillogenic pathway.

Apolipoprotein AI amyloidosis

Apolipoprotein AI (apoAI) is a 243-amino acid protein synthesized in the liver and small intestine and secreted into plasma as lipoprotein particles. It is the principal protein component of high density lipoproteins (HDL),

and its biological functions include activation of lecithin-cholesterol acyltransferase, the enzyme responsible for cholesterol esterification in plasma. ApoAI binds and transports plasma lipid and increases cholesterol efflux from peripheral tissues in a process called reverse cholesterol transport [62]. It has been calculated that approximately 4% of plasma apoAI is not HDL-bound and circulates there as free protein [63]. Extraordinary efforts have been made to obtain a 3D structure of apoAI, and crystallographic data obtained with the lipid-free form of apoAI (residues 44–243) have shed new light on the structure of the lipid-binding domain. The molecule consists of a pseudocontinuous, amphipathic α helix punctuated by kinks at proline residues, and it adopts an overall shape similar to a horseshoe [64]. This exchangeable lipoprotein most likely exhibits different structural conformations in the lipid-free state versus the lipid-bound state, and the transition between the two can be extremely important for lipid binding, interaction with a putative receptor, protein catabolism and pathological misassembly in amyloidosis. It has been suggested that the transition between the lipid-bound and lipid-free state could pass through a molten globular state, and the interaction of hydrophobic residues with sterols could strongly favor the process of achieving a completely helical and stable folded state [65]. Amyloidosis caused by apolipoproteins was first described 10 years ago by Benson's group [66], and so far six different mutations in the apoAI gene have been correlated with various forms of hereditary amyloidosis. Three of these mutations are the result of a single-nucleotide change that gives rise to an amino acid substitution in the N-terminal domain of the protein and confers an extra positive charge on the molecule [67–70]. One is a deletion [71], and one is a deletion/insertion mutation; both result in an increase in the isoelectric point of the molecule [72]. A French family presenting a point mutation in exon 4 of the apoAI gene, responsible for a substitution of Leu90 with Pro, was reported very recently [73], and in this case a change in the isoelectric point was not directly evident. But the ability of Pro to break the helix can have an overall destabilizing effect.

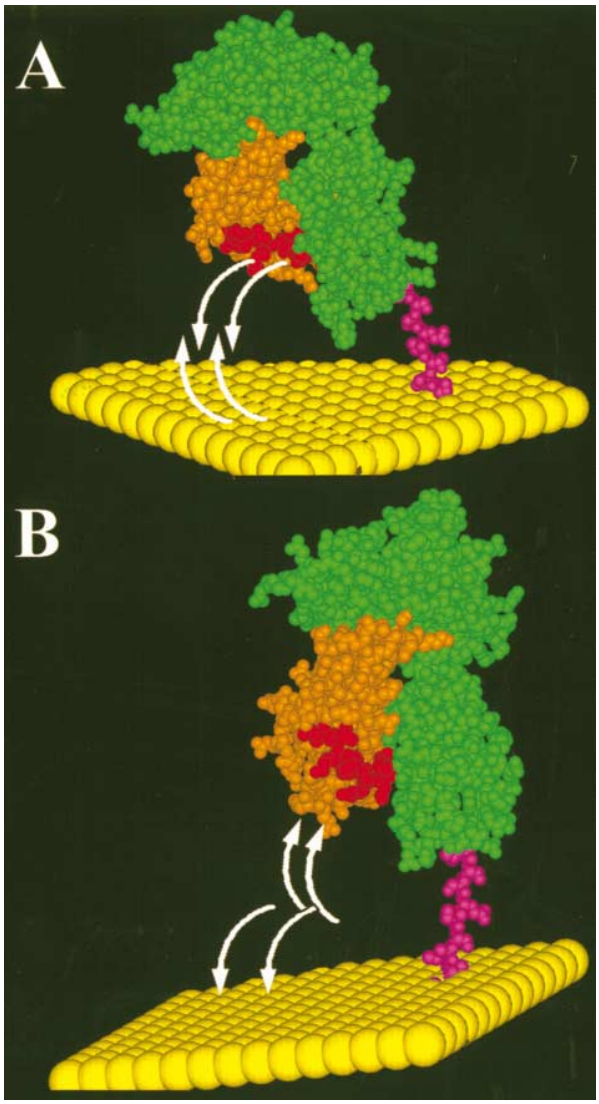

Figure 3. A hypothetical model of the MHCI complex bound to a cell membrane. Yellow spheres represent the polar heads of the phospholipids, whereas the MHC heavy-chain atoms are colored in green, the peptide connecting the heavy chain to the membrane in violet, β2-m in orange and the antigenic peptide 92–99 in red. Atomic coordinates of the MHC complex were taken from the Brookhaven Protein Data Bank, set 3HLA [111], whereas the connecting peptide (aa 271–284 according to the numbering system of the crystal structure) was built as a β-strand in an extended conformation. In panel *A* MHCI is oriented with its long axis nearly perpendicular to the phospholipid bilayer, and in this orientation peptide 92–99 of β2-m is hidden between the heavy chain and the membrane surface and therefore not accessible for the interaction with the antibody. In panel *B* the simple rotation of the flexible peptide that anchors MHCI to the membrane produces a reorientation of the complex with respect to the membrane, making epitope 92–99 accessible to the antibody.

A peculiar form of hereditary systemic amyloidosis has also been reported very recently by Prof. Pepys's group [74]. In this case, amyloid deposits of fibrillar apoAI were found in the skin, kidney and spleen, but no mutations were found in the 82-residue N-terminal polypeptide isolated from the fibrils. This case offers a strong demonstration that the N-terminal domain of apoAI is potentially amyloidogenic in the wild-type species. The capacity of wild-type apoAI to create fibrils has been shown in humans in association with atheromatous plaques in the intima of the aorta [75], and it has been documented in dogs in their 2nd decade that develop amyloid deposits in the pulmonary arteries at the level of the intima and media [76, 77].

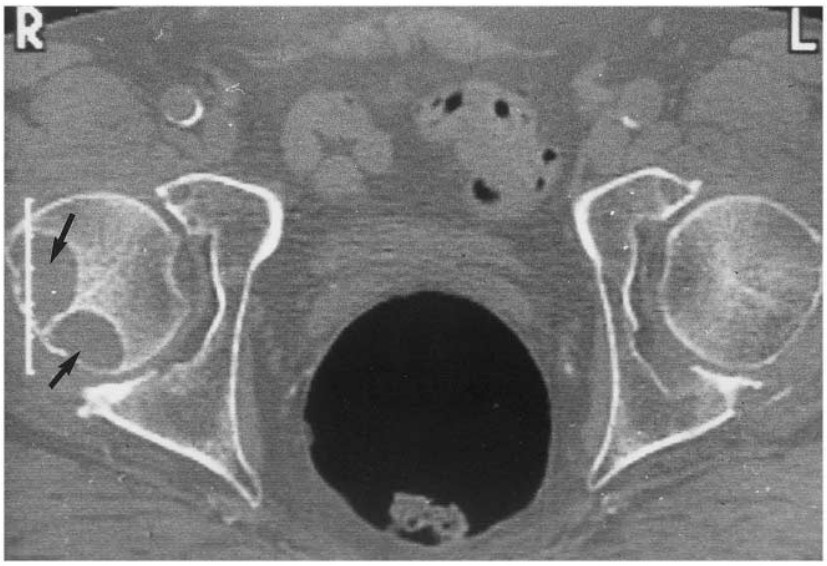

Figure 4. Computed tomography (CT) scan of the hip. The arrow indicates amyloid erosions of the right femur. Reproduced with permission from Bardin et al. [112].

The property of fibrillar apoAI, common to both the forms deriving from mutated species as well as the nonmutated ones, is the size of the polypeptide chain that constitutes the fibril. In fact, structural studies including N-terminal amino acid sequence and mass determination by mass spectrometry have shown that apoAI fragments corresponding to the first 80–100 residues of the proteins are the main constituents of the fibrils in the hereditary familial forms. This finding was confirmed in apoAI fibrils identified in the aortic intima in association with the atheromatous plaques in at least one case in which an investigation of chemical properties revealed the presence of the 69 N-terminal residues [75]. The fibrillar apoAI associated with atherosclerotic plaques derives from a wild-type species, but deletion of a Lys residue in position 107 was detected in a case in which the phenomenon of intimal amyloid deposit was particularly severe [78]. Likewise, in apoAI amyloidosis affecting aged dogs, the chemistry of the fibril protein suggests that the main constituent is the N-terminal domain of the protein, in this case a 71-amino acid residue identical to the human counterpart with the exception of an extra Pro in position 4 [76]. The secondary structure of this N-terminal domain as determined from the limited crystallographic data of apoAI 44–244 and from structural prediction should be roughly an α helix. The fibrillogenic process must therefore require a massive conversion of the secondary structure from α to β in this apoAI domain.

The role played by the N-terminal peptide 1–43 and the C-terminal portion 187–243 of apoAI in structure, folding dynamics and protection from proteolysis has recently been thoroughly investigated by Rogers et al. [79]. Removal of the first 43 N-terminal residues destabilizes the molecule (Cm in urea = 1.9 M) as compared with the wild-type ($Cm = 2.64$ M) and C-terminal-deleted species ($Cm = 2.6$ M). Removal of the first 43 N-terminal residues has the effect of increasing the percentage of the secondary helical structure and of modifying susceptibility to chymotrypsin digestion. Whereas the wild-type and the C-terminal-deleted species are quite resistant to proteolysis, the form truncated at the N-terminal is more effectively proteolized. All the amyloidogenic mutations reported so far are located in the first 110 residues of the molecule, but it is not known whether the effect of these mutations is overall protein destabilization or tertiary structure modifications similar to those described in the N-terminal-deleted form. Besides a structural comparison between the wild-type and the variants, it would be extremely fruitful to extend the structural analysis to the lipid-bound and lipid-free states and to monitor their susceptibility to proteolytic action. These experiments would allow us to observe the phenomenon of apoAI fibrillogenesis in a dynamic

perspective that appears to be particularly appropriate for this 'exchangeable' lipoprotein. It would not be surprising if the best substrate for the creation of the amyloidogenic N-terminal peptide was the molten globular state that apoAI passes through on its way to accomplishing its exchange function. The mutation could act by changing the rate of lipid exchange and by modifying the kinetics of the conformational changes associated with various phases of lipid transport. It could be hypothesized that in apoAI as well, a folding intermediate must become highly populated in order to open the fibrillogenic pathway that in this case could require the proteolytic event. The levels of apoAI and HDL are frequently lower in these amyloid patients than in normal subjects, and abnormalities in the metabolism of apoAI variants Gly26Arg have been reported [80]. These experiments were conducted in humans using a radiolabelled mixture of the wild-type and the variant, and both species were rapidly removed from plasma, indicating that the presence of the mutation accelerates the catabolism of HDL particles in which a mixture of wild-type and variant are represented. Abnormalities in apoAI metabolism are not, however, a peculiar feature of the amyloidogenic apoAI variant; they have been described for other nonamyloidogenic apoAI mutations, like that of apoAI Milano (Arg173Cys), in which the mutant is rapidly removed from plasma [81].

Recent studies carried out in the animal model with recombinant apoAI Gly26Arg [82] confirmed the high catabolic rate of this amyloidogenic species. In this study, density gradient ultracentrifugation analysis showed an increased association of amyloidogenic apoAI with the denser HDL_3 particles, whereas apoAI wild-type was primarily associated with the HDL_2 fraction. The reduced association between the variant apoAI and HDL_2 explains its rapid catabolism, because once apoAI dissociates from HDL, it is quickly proteolized [83]. The interaction between apoAI and other proteins not directly involved in lipid metabolism but relevant for the membrane structure, like annexin I and VII, has been documented [84]. The putative influence of the interaction with these proteins on the pathological phenotype of apoAI amyloidosis is not known but could represent a promising research topic for future.

Fibril function

We have so far analyzed the biological function, structure and folding pathway of four amyloidogenic proteins and looked at possible connections between their properties and the final common pathological outcome: the formation of amyloid fibrils. Despite the involvement of different proteins, amyloid fibrils have the same molecular features [85], and the primitive function performed by the protein precursor is abolished. However, new biological functions are acquired by this protein polymer, and somehow these functions (binding to SAP, interaction with glycosaminoglycans and cell toxicity) contribute to the persistence and progression in the tissue of the pathological protein aggregate. While interaction with SAP [86] and glycosaminoglycans [87] is well documented in all types of amyloidosis, cell-fibril interaction has been extensively investigated only in AD. In this type of amyloidosis the protein that makes fibrils, called amyloid β protein (Aβ), is a polypeptide of 39–43 residues that derives from the Alzheimer amyloid precursor protein (APP). The Aβ amyloid polypeptide is generated by two distinct proteolytic events referable to β and γ secretase [88]: two proteases whose chemical identity has not yet been elucidated. The Aβ protein easily aggregates and creates fibrils in vitro, an extensively investigated phenomenon that shows peculiar structural and kinetical features [89].

The Aβ aggregates can induce neurotoxicity, loss of presynaptic terminals and the development of dystrophic neuritis [90]. Several studies have demonstrated that the neuron insult caused by Aβ correlates with the formation of the aggregate, and the degree of toxicity is somehow dose dependent on the level of aggregation [91].

The mechanism of neurotoxicity has been well investigated and appears to be associated with the activation of certain components of intracellular signal transmission through selective induction of genes like c-jun, c-fos, jun B and fos B [92]. There is also strong evidence that the cellular response to Aβ fibrils documented in the in vitro model is very similar to its in vivo counterpart: c-fos and c-jun are highly expressed in the neurons of AD patients [93], and the mechanism of cellular death shows typical features of apoptosis [94]. The role played by jun kinase in neuron apoptosis is well documented, and it has been hypothesized that Aβ can activate this kinase pathway, which primes a feedback circuit in which jun kinase phosphorylates c-jun and induces the expression of its own gene [92]. The activation of these protein kinases, caused by Aβ fibrils, most likely has the effect of hyperphosphorylating protein τ, which physiologically interacts with tubulin, binds to microtubules and promotes microtubule assembly; in the covalently modified state τ aggregates in paired helical filaments and contributes to the formation of the pathognomonic neurofibrillar tangles.

The phosphorylation of protein τ has the effect of reducing its ability to facilitate the correct assembly of microtubules [95], and it has even been suggested that

the abnormal phosphorylation of τ in AD can cause microtubule depolymerization, impaired axonal transport and neuronal degeneration. It is worth emphasising that the injection of fibrillar Aβ into cortices of aged rhesus monkeys results in τ phosphorylation, but this effect is not obtained with injection of the soluble form of Aβ [96].

The cause-effect relationship between fibrils and cell toxicity has therefore been established, but it is not easy to understand the mechanism by which this insoluble fibrillar material can transmit toxic signals to cells. So far the molecular structure of the aggregate that is most effective in exerting its toxicity has not been otherwise defined, expecially considering that frequently the amount of amyloid deposition in AD as in other amyloidoses is not correlated with the clinical and pathological severity of tissue damage [97].

In an effort to standardize the dose/toxicity correlation as well as elucidate the putative contribution of other proteins in this phenomenon, a study was recently published with detailed characterization of the dimensions of the β-protein aggregates that mediate the neurotoxic signal [98]. In their paper the authors demonstrated that a molecular entity called ADDL (Aβ-derived diffusible ligand) was able to kill mature neurons in central nervous cell cultures at nanomolar concentration. Analysis of ADDL by atomic force microscopy revealed that toxic oligomers have a globular structure of approximately 5–6 nm in size, and acrylamide gradient gel electrophoresis clearly identified two main species of 27 and 17 kDa. ADDLs probably bind to cell surface sites which contain particular domains of specific proteins. Indeed, exposure of cells to trypsin has the effect of inhibiting ADDL binding and preventing consequent cell toxicity.

ADDL toxicity was found to depend also on expression of *fyn*, a tyrosine kinase protein linked to apoptosis and overexpressed in AD. The authors tested the effect of ADDL on brain slices of $fyn-/-$ and $fyn+/+$ transgenic animals and found that the *fyn* knockout animals are protected from ADDL toxicity. These findings suggest that oligomers of Aβ 1–42 can interact, at a very initial level of aggregation, with a specific cell receptor involved in starting the apoptotic pathway. Other cell surface neuronal receptors have been reported to bind aggregated β amyloid. The p75 neurotrophin receptor binds monomeric β peptide with an affinity higher than that of the aggregated β peptide, but only in this last state does the peptide induce a cellular toxicity [99]. Aggregated β amyloid binds the cell surface neuronal receptors for glycation end products [100] leading to generation of oxygen- and nitrogen-reactive radicals that damage the neurons.

Alteration of cellular functions in AD can, however, be trigged by intracellular Aβ peptide, whose presence inside the cell in different states of aggregation has been documented [101]. Aβ can bind to and inhibit the biological function of intracellular molecules like the polypeptide called ERAB by Yan et al. [102], a polypeptide which is normally present in neurons and overexpressed in a complex with Aβ peptide in the case of AD [102]. Hydroxysteroid dehydrogenase activity has been postulated for this molecule on the basis of its amino acid sequence; furthermore, it contains a structural motif typical of a NAD binding site and a putative active center of the steroid binding domain. It is possible that ERAB normally functions in cellular metabolism and biosynthesis and that the β peptide modulates its activity and rearranges its distribution within the cell.

Definition of the structural characteristics of the toxic species of amyloid fibrils is much less advanced in other types of amyloidosis. Data recently reported by Lundgren and co-workers demonstrate that amyloidogenic TTR, when converted into fibrils, also displays cytotoxic activity [103], and it has been established that islet amyloid polypeptide (IAPP) fibrils have a toxic effect on Langerhans β cells in non-insulin-dependent diabetes [104]. Nevertheless, certain discrepancies exist between experiments conducted in different laboratories with different IAPP preparations. Recent data reported by Janson et al. [105] suggest that the cytotoxic species are made up of free or small early aggregates of islet amyloid polypeptide. By contrast, mature human islet amyloid polypeptide solutions containing organized fibrils are seldom associated with features of membrane damage and cell death. In this light, the findings reported by Clark and co-workers [106] on the extreme variability, in terms of toxicity, between different IAPP batches that can be caused by heterogeneity in the level of peptide aggregation, appear to be more understandable. Therefore, in this type of amyloidosis, as in AD, the mechanism of cell toxicity seems to be mediated by a defined molecular entity created by oligomers of limited dimension that specifically interact with a defined cell receptor.

Study of the cell toxicity diplayed by amyloid fibrils has brought to light new mechanisms of protein-protein interactions in the transmission of messages to cells that will probably be rapidly extended to all form of amyloidosis. The demonstration that certain compounds like Congo red [107], anthracyclines [108] and rifampicin [109] can avidly bind the fibrils and, without modifying their overall structure, annihilate their cytotoxic power, offers useful tools for this research and opens realistic therapeutic perspectives.

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