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Formation of amyloid-like aggregates through the attachment of protein molecules to a Congo red scaffolding framework ordered under the influence of an electric field

Research Article

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Abstract: This study describes a technique which makes it possible to introduce the amyloid-like order to protein aggregates by using the scaffolding framework built from supramolecular, fibrillar Congo red structures arranged in an electric field. The electric field was used not only to obtain a uniform orientation of the charged dye fibrils, but also to make the fibrils long, compact and rigid due to the delocalization of pi electrons, which favors ring stacking and, as a consequence, results in an increased tendency to self-assemble. The protein molecules (immunoglobulin L chain lambda, ferritin) attached to this easily adsorbing dye framework assume its ordered structure. The complex precipitating as plate-like fragments shows birefringence in polarized light. The parallel organization of fibrils can be observed with an electron microscope. The dye framework may be removed *via* reduction with sodium dithionite, leaving the aggregated protein molecules in the ordered state, as confirmed by X-ray diffraction studies.

Keywords: *Congo red • Amyloid formation • Electric field*

1. Introduction

The *in vivo* formed protein aggregates with an ordered structure are easily anchored in tissues which have exposed repeatable structural receptor motifs. The damaging effects and the subsequent serious clinical manifestations are the result of this phenomenon [1-5]. Understanding this phenomenon and preventing it are among the main challenges in modern medicine. It seems probable that these aims may be achieved by researching methods that enable the controlled formation

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of protein aggregates for model studies [6-13]. The technique described in the present paper is based on the application of fibrillar, supramolecular structures formed by Congo red (or other dyes of the Congo red family), which can act as scaffolding matrices [14]. However, the adaptation of Congo red to the scaffolding role requires the fibrils of the dye to be rigid and properly oriented. As is to be expected, compact and rigid fibrils may be obtained by the close stacking of dye rings in a face-to-face manner [15-20]. The intensive self-assembly may be engineered by decreasing the repulsive properties of dye molecules, which depend on the charged substituents and π electrons of the rings [21-23]. This may be achieved *via* the protonation of amino groups at low pH values and/or screening of charges at high salt concentrations. However, the self-assembly of dye molecules may also be induced efficiently using the influence of an electric field. This effect can be achieved by changing the protonation of the substituents, but more likely it occurs as a result of the direct influence of the electric field on π electrons' delocalization. Supramolecular structures are often susceptible to significant alterations caused by external factors, through even slight changes of the properties of the assembling molecules or through affecting the already assembled dye forms [24-27].

The created compact fibrillar dye structures may bind protein molecules and favor their aggregation by allowing them to concentrate locally and come into close contact. Since the partition of highly compact dye fibrils by proteins is impossible, the alteration of the protein binding mechanism that occurs in neutral or alkaline solutions is thought to be necessary [28-30]. It may be supposed that protein molecules gather on the thread-like micellar dye entities, producing large formations rather than populations of individual protein molecules carrying Congo red particles. The inducing effect of the electric field on the compactness of the Congo red supramolecular structures and the application of this effect for the formation of amyloid-like protein aggregates by means of the scaffolding technique is presented in this paper.

2. Experimental procedures

2.1. Materials

All reagents used were of analytical grade. Congo red (97% purity) from Aldrich Chemical Co. Immunoglobulin L (λ) chain was obtained from the urine of a myeloma patient. It was purified by salting out followed by Sephacryl S-300 gel filtration. Ferritin and plasma albumin (crystallized bovine serum albumin) were purchased from Sigma (USA).

2.2. Agarose gel electrophoresis of Congo red at different concentrations

Agarose gel (1%) electrophoresis of Congo red was performed in 0.05 M acetate buffer of pH 5.5 at 100, 200 and/or 300 V. Concentrations of Congo red used were: 0.1, 0.25, 0.5, 1.0, 2.0, 5.0, 7.5 and 10 mg mL^{-1}.

2.3. Measurement of pH in DMSO/water solutions containing Congo red

The measurement of hydrogen ion concentration in solutions containing DMSO, designed to follow the process of Congo red self-assembling upon the increasing content of water, was performed using the chinhydron electrode. The mixture of DMSO and 0.2% NaCl in water (molar ratio water:DMSO equal 2:1), containing Congo red at 2 mg mL^{-1} was used as the initial solution. The pH was adjusted to 4.9. Then, 0.2% NaCl solution, or Congo red (2 mg mL^{-1}) solution in 0.2% NaCl, was added gradually to increase the water:DMSO molar ratio up to 10:1. At each step the pH of the solution was measured. The experiment was performed in water cooled vessel and pH was measured at 22°C.

2.4. The Congo red protein complexation test

The immunoglobulin L chain λ (capable to form complexes with Congo red spontaneously) and Congo red solutions (both in 0.05 M acetate buffer of pH 5.3) were mixed to give final L chain concentration 6.9 mg mL^{-1}. The following protein:dye molar ratios were used: 1:0.125, 1:0.25, 1:0.5, 1:1, 1:2, 1:4, 1:6 and 1:12. Relative mobilities of the complexes in agarose gel electrophoresis (at pH 5.3) were measured versus bromophenol blue.

2.5. Free electrophoresis conditions used to induce protein aggregation

The aggregation of proteins induced by the electric field was performed in glass tubing (2 mL volume) with a small chimney in the middle, allowing contact with the reaction mixture during the process. The system was cooled to keep the samples at room temperature. Both ends of the tubing were closed with cellulose membranes and separated from electrodes by the sufficiently long buffer barrier to avoid any pH alteration in the reaction chamber.

The mixture containing the L chain (30 mg mL^{-1}) and Congo red at the dye:protein molar ratio 4:1 was kept at 300 V. The direction of the electric field was changed every 10 minutes. The samples (7 μL) were collected every 15 minutes and then tested for the increasing content of higher molecular weight fractions by 15% polyacrylamide gel electrophoresis (Tris-glycine buffer pH 8.3, 90-120 V).

Ferritin at 2.5 mg mL^{-1} (in 0.05 M acetate buffer, pH 5.3), was heated for 10 minutes at 65°C and then added to the Congo red solution (3 mg mL^{-1}, in the same buffer) and kept in the at 300 V for 15 minutes.

2.6. Albumin binding to rigid, supramolecular Congo red

Solutions containing 0.2 mg of Congo red in 0.15 M NaCl (pH 8.2) were mixed with 0.5 mL portions of swelled Bio-Gel P-10 (Bio-Rad) grains. Congo red adsorbed to the grains was then acidified by the addition of 0.2 mL of 0.1 M acetate buffer (different pH). Suspensions of grains were centrifuged to remove the excess solution. Prepared in this way, Congo red carrying grains were mixed with 0.1 mL solution containing 1 mg of bovine serum albumin in 0.1 M acetate buffers of different pH. After 10 minutes of incubation the samples were centrifuged and the supernatants were analyzed to determine the dye : protein ratio. Congo red concentrations were measured spectrophotometrically (absorption coefficient k_{489} = 50.43 mL mg^{-1} cm^{-1}). Protein concentrations were determined by dot staining of supernatant samples with bromophenol blue. In the control experiment 0.1 mL portions of albumin-Congo red complex (formed in 0.15 M NaCl, pH 8.2) were mixed with 0.5 mL samples of Bio-Gel P-10 grains, previously equilibrated with 0.1 M acetate buffers of different pH. After 10 minutes the samples were centrifuged. The dye and protein concentrations in supernates were determined as described above. In both experiments the initial Congo red:albumin molar ratio equaled 19.

2.7. Microscopic analysis

Electron microscopy studies were performed with a Philips RM 300 using uranyl acetate for negative staining. A Zeiss Axioplan II with polarization equipment was used for light microscopy analyses.

2.8. X-ray fiber diffraction

X-ray diffraction images of amyloid and lyophilised protein samples mounted in the nylon loop were taken on a Nonius KappaCCD diffractometer using graphite monochromic MoKα radiation (λ=0.7107 Å, 55 kV, 30 mA). Radial distribution of relative intensity was determined by integration of diffraction images using POWDERIZE procedure in the diffractometer software.

2.9. Molecular dynamics simulation

In the first part of the project fully solvated Molecular Dynamics (MD) simulations of myoglobin monomers were performed, with the aim of preparing its structure for the subsequent docking of a Congo Red (CR) micelle. The crystal structure of myoglobin was obtained from the Protein Data Bank [31] (PDB code - 2V1K.pdb). This particular structure was chosen because of its high resolution (1.25 Å). Since the Congo red micelle is likely to occupy the hydrophobic heme binding pocket, two simulation systems were used: an *apo* form with the heme molecule removed and a native, *holo* form. The latter was used as a control to assess the degree of structural disruptions caused by the removal of the heme.

Prior to simulation, each system was immersed in a rectangular box of TIP3P waters. The position of each system in its box was optimized by the Simulaid program [32] to yield the smallest possible volume of the box with the minimal primary atom - image atom distance set to 25 Å. Each system was simulated at three different temperatures – 300 K, 350 K and 400 K for 5 ns in NPT ensemble. Higher simulation temperatures were used to enhance conformational sampling and to obtain a 'molten globule' state which is likely to bind the micelle.

The resulting post-MD structures of the apo form were used as templates for the docking of CR micelles. The micelle of CR was prepared as described earlier [14]. In the docking process the CR micelle was manually placed to maximize its interactions with the residues of the heme binding pocket. Several different orientations were tried with the general constraint that the micelle must be able to propagate after protein binding, in accordance with experimental observations. Subsequently, each complex was relaxed and optimized using a Monte Carlo (MC) simulation technique with scaled nonbonding interactions. For one complete relaxation set, we have run ten Monte Carlo simulations with a scaling factor increasing from 0.1 in the first run to 1.0 in the last run. We have introduced energy scaling between the protein and the ligand molecules to avoid extremely high potential energy values in the early stages of the simulation that arise from the substantial overlap of protein and ligand atoms. Such high-energy values would potentially lead to the irreversible disruption of the native structure. During all MC simulations all amino acids not involved in the interactions with the ligand were kept fixed.

At the end of the MC relaxation process we decided to choose two complexes for further MD simulations. The criteria for this choice were the low overall energy of the complex, the ability of the micelle to propagate without disrupting the complex and the relatively intact geometry of the micelle. The chosen complexes had radically different placements of the micelle with respect to the protein, thus indicating that several alternative, low-energy placements of the micelle are possible.

The MC-relaxed complexes were immersed in rectangular boxes of water and were subjected to 5 ns

long MD simulations at three different temperatures 300 K, 350 K and 400 K. During the simulations, structures were collected every picosecond and were used in the post-dynamical analyses.

All MD simulations were performed using NAMD2.6 [33] with the charmm22 all-atom forcefield [34], extended to accommodate a CR molecule [35]. The solvent was represented by TIP3P water model and NPT ensemble was used with the pressure fixed at 1atm. All bond lengths between hydrogens and heavy atoms were fixed using the SHAKE algorithm, which allowed a time step of 2 fsec.

All MC calculations were performed in vacuum using CHARMM 31b1 [36].

3. Results and discussion

3.1. Congo red self-assembly and charge relationship

As the dye concentration increases, its electrophoretic migration rate increases surprisingly (Fig. 1). The increased electrophorteic mobility indicates that the more tightly packed dye populations which are formed as a consequence of the increasing concentration exhibit more negative charge in the electric field. The relation between self-assembly and the charge suggests that the electric field may be used to interfere with the process of formation of the supramolecular dye structures. However, the increasingly negative charge of Congo red which is observed in electrophoresis at higher concentrations of the dye, indicates that favorable association of molecules occurs not so much through spontaneous self-assembly, but through self-assembly induced by the electric field.

In the absence of the electric field, self-assembly leads to an increase in pH and the subsequent decrease of the dye's acidity (Fig. 1, inset). This effect was observed after the change of pH during the transition from the single molecular state to the self-assembled Congo red form in a solution containing DMSO. This transition was performed in conditions in which the content of DMSO gradually decreased [37]. This may indicate the dye's amino groups' increasing tendency to protonate. The opposite effect, *i.e.,* when the charge of the dye becomes increasingly more negative with the increase in its concentration during electrophoresis, seems to derive from the delocalization of π electrons due to the influence of the electric field. The spectrum of Congo red electrophoretic mobility at its different concentrations (see Fig. 1) was found to change with increasing voltage, but in a disproportionate way (Fig. 2). The increase of Congo red mobility (evaluated versus the migration of bromophenol blue) is more significant for Congo red solutions of lower concentrations, *i.e.,* those containing dye molecules of lower degree of self-assembly. The uneven/irregular acceleration of dye migration, which favors the dye in solutions of lower degree of self-assembly,

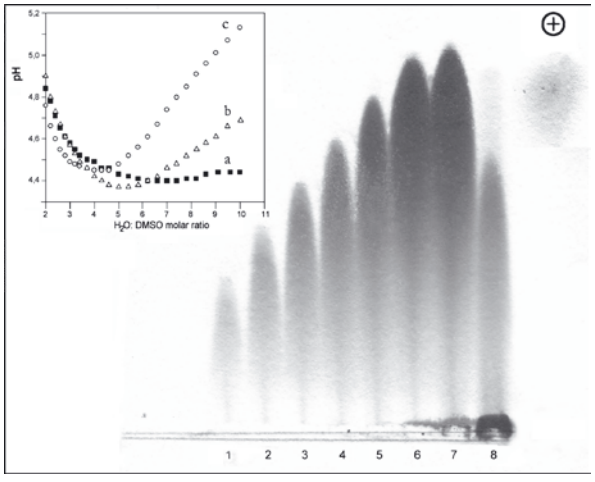

Figure 1. Agarose gel electrophoresis of Congo red demonstrating the different mobility of the dye at different concentrations (0.1, 0.25, 0.5, 1.0, 2.0, 5.0, 7.5 and 10 mg mL^{-1}, for samples 1-8, respectively). Inset – the change of pH in a DMSO-water solution of Congo red upon a gradual increase of water (0.2% NaCl)/DMSO molar ratio in the range which covers the transition of the dye from monomeric to self-assembled forms (which occurs at about 5-6 water:DMSO molar ratio) [37]. The pH of all samples was adjusted to 4.9 prior to starting the dilution of DMSO. (a) control: DMSO/water solution; (b) Congo red concentration decreased upon dilution; (c) Congo red concentration maintained at a constant level by adding a water solution of the dye to the sample.

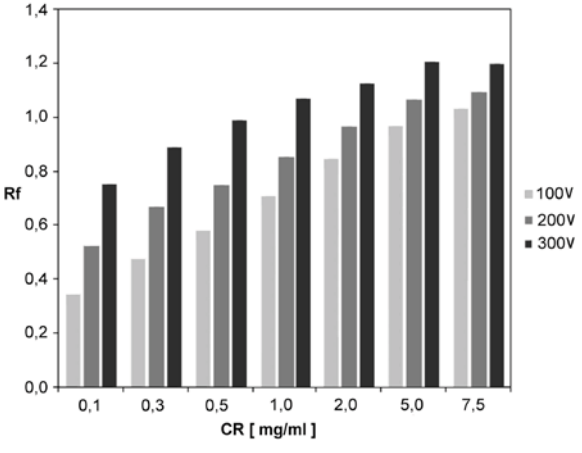

Figure 2. The uneven increase of Congo red electrophoretic mobility in solutions of different dye concentration observed upon increasing the voltage. The more significant acceleration effect concerns Congo red solutions of lower concentration and lower degree of self-assembly, and therefore a higher propensity to change.

convincingly points to the electric field as the reason for this phenomenon. The self-assembly enhanced by the electric field makes the supramolecular fibrillar dye structures more compact and rigid, and thus suitable for the scaffolding role. The dye framework which is formed as a result of shifting the self-assembly equilibrium toward more extended structures seems to be stabilized by protein complexation, eventually producing ordered solid aggregates.

3.2. Dye-protein complexation conducted in an applied external electric field

The ordered amyloid-like protein aggregates were formed *via* complexation of proteins with Congo red in conditions strongly favoring self-assembly of the dye molecules (low pH, electric field). The affinity and subsequent complexation of the dye and studied proteins in these conditions were confirmed experimentally [14]. In the present study it is evidenced using immunoglobulin L chain lambda, and ferritin. The occurrence of complexation is proved by the fact that the direction of electrophoretic migration (at pH 5.3) of the L chain changes as the dye/protein ratio increases, until the charge of the dye prevails (Fig. 3).

The proteins used in the investigations were either structurally unstable (abnormal L chain λ), or heated until they became partly unfolded (ferritin) in order to allow them to aggregate, as is commonly done for amyloid formation [38,39]. Both proteins were initially soluble. For complexation, the proteins were mixed with Congo red, just before initiating the incubation at 300 V. A large fraction of the initially soluble protein-dye complex precipitated as sheets exhibiting birefringence in polarized light. Samples of the still soluble mixture were collected every 15 minutes, and then tested by electrophoresis in a high-density polyacrylamide gel (15%) to determine whether the concentration of fractions with molecular weight higher than that of the L λ chain had increased. No further modification of the aggregation state in samples seems possible with polyacrylamide-gel electrophoresis, since the Congo red scaffolding framework undergoes destruction due to the change in pH (to 8.3) and is separated from the protein in the dense sieve bed. In addition, the applied voltage is significantly lower (about 90 V). Fig. 4 confirms the predicted intensified formation of more aggregated forms of the complex, as indicated by the increasing content of still soluble aggregates. They can be seen as the streaky, slowly migrating fractions with molecular weight higher than that of the protein used for the complexation with Congo red.

Complexes of partly unfolded proteins with dye fibrils are formed in spite of the mode of complexation,

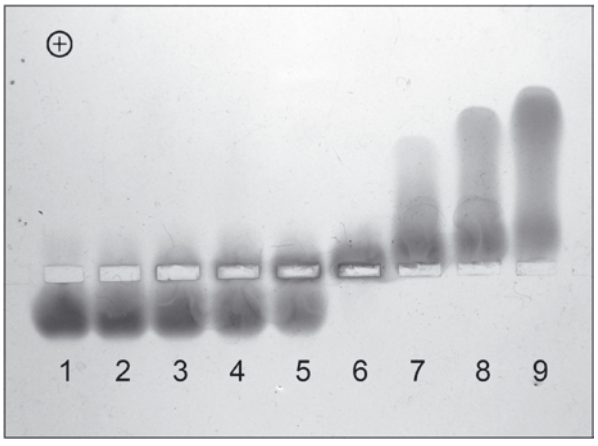

Figure 3. Congo red immunoglobulin L chain λ complexation proved by changing the direction of protein migration in agarose gel electrophoresis (performed at a pH of 5.3) upon increasing the Congo red to protein ratio. (1) control (without Congo red); (2)-(9) Congo red:protein molar ratio equal 1:0.125, 1:0.25, 1:0.5, 1:1, 1:2 , 1:4, 1:6 and 1:12, respectively. The gel was stained with bromophenol blue to visualize proteins after decolorizing Congo red *via* reduction (sodium dithionite).

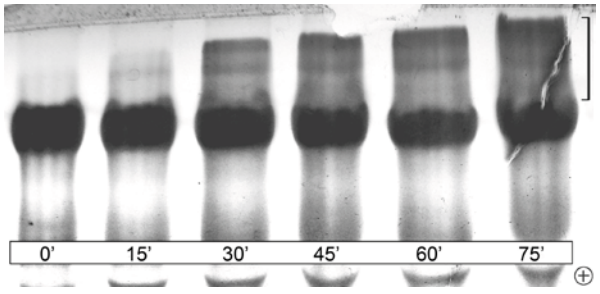

Figure 4. The electrophoretic separation (15% polyacrylamide gel, pH 8.3) of samples containing the immunoglobulin L chain λ and the increasing content of its aggregates (marked by the bracket), formed as the L chain was driven to aggregation in the presence of Congo red at 300 V, pH 5.5. 0' – control sample. The gel was stained with Coomassie Brilliant Blue G-250 after removal of Congo red *via* sodium dithionite reduction.

which is altered due to significantly increased rigidity of supramolecular-structures. The partition of compact micellar dye structures by individual protein molecules, which occurs in neutral or alkaline solutions, is no longer possible [28,40]. The attachment of protein molecules to dye threads and formation of large complexes is rather to be expected in these conditions. Due to the poor plasticity of the compact and rigid dye fibrils, the fitting requirement is more likely to concern the protein rather than the dye. To verify this prediction, the albumin-Congo red complexation was analyzed after the compactness of dye particles had increased upon decreasing the pH. Serum albumin binds supramolecular Congo red as the native protein [41]. Its interaction with the dye is more specific than that of amyloidogenic, partly

unfolded proteins, and hence it seems to represent a convenient, restrictive model for testing the capability of binding the dye with the altered, highly compact structure. About 17 Congo red molecules are bound to the albumin molecule in neutral or alkaline solutions, most as the self-assembled dye portion [41]. In order to test the complexation at different pH values (including the range in which Congo red solubility is low), the dye was used in the form adsorbed to the BioGel grains. As it is fully accessible in this form, weakly adsorbed dye was easily taken up by albumin. When the complex was formed in alkaline solutions, it remained unchanged despite its acidification to a pH of 4.5. In contrast, the binding of the dye by albumin at low values of pH (near the pK of the dye amino group [14]) becomes significantly less efficient (Fig. 5).

The decreased capability of binding the dye, down to the level of three molecules, may be interpreted as the loss of albumin's specific capability to bind the dye in its supramolecular, rigid form, inadequate to the albumin binding site. This result confirms the anticipated difficulties in dissociating strongly assembled dye micelles into monomers and oligomers. However, it also makes it possible to understand the observed complexation of partly unfolded proteins with Congo red simply as the attachment of protein molecules to rigid, indivisible dye fibrils. Thus, large particles of the complex are formed, rather than populations of individual dye-carrying protein molecules.

Two complexation forms are basically likely to appear considering the protein attachment to the rigid dye fibrils. In the first one mostly hydrophobic portions of dye involving benzidine rings are engaged in complexation, and the second one involves the engagement of naphthalene rings, which are more available for complexation. Fig. 6 presents the latter, more probable model, as predicted with calculations using apomyoglobin as an exemplary protein.

The fibrillar, parallel organization of the material can be seen in Fig. 7, which presents the electron microscope picture of the L λ chain–Congo red complex. The independently analyzed Congo red (without the protein) particles in the arrangement induced by the electric field, reveals that it also has a fibrillar structure (Fig. 7, inset). The protein aggregates arranged in the scaffolding framework form plates of fibrillar nature rather than independent fibrils.

Although most of the precipitated protein-dye material is ordered, the degree of its organization differs. Some pieces are particularly well-arranged and show a higher order of molecular packing, and become near crystal-like (Fig. 8) [14,42]. They are easily differentiated in polarized light as bright regions within larger particles (Fig. 8, inset).

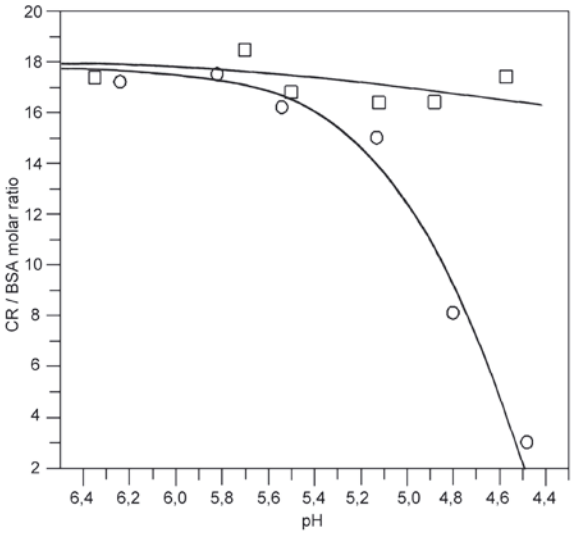

Figure 5. Changes of the propensity of albumin to form complexes with Congo red when decreasing the pH. o - Albumin was added for complexation with Congo red previously adsorbed to BioGel P-10 beads, which were then equilibrated with appropriate buffers. □ - Control sample - albumin was first complexed with Congo red at pH 8.2, and then exposed to Bio-Gel beads at different pH values. The experiment demonstrates the higher stability of the albumin-Congo red complex formed in alkaline solutions.

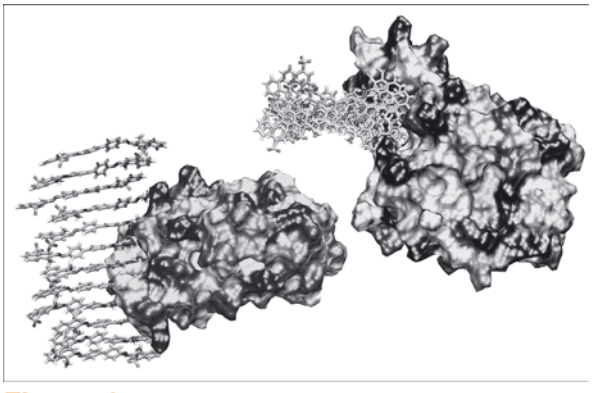

Figure 6. The computational model of apomyoglobin attached to the fragment of rigid Congo red fibril. The figure shows two different projections of the same complex

Fig. 9 shows the ferritin-Congo red complex obtained in a similar way (electric field, pH 5.3). The complexes formed with this protein, arranged by the dye framework, are visible under the electron microscope without negative staining as plates composed of large, organized, parallel fibres of metal-bearing molecules. The staining of ferritin-Congo red complexes with uranyl cations exposes the arrangement of negatively charged Congo red fibrils seen in the picture as black streaks among protein fibres (Fig. 9, inset).

In order to determine whether protein molecules assembled on Congo red fibrils had already aggregated

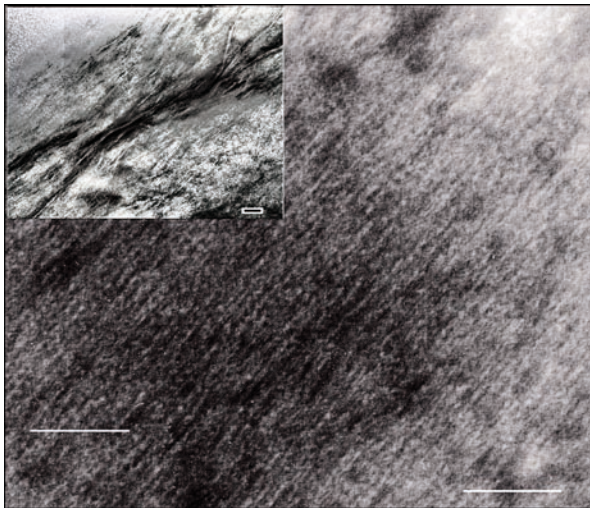

Figure 7. Electron micrograph of aggregated immunoglobulin L chain λ - Congo red complexes, arranged under the influence of the electric field. The inset shows the fibrillar organization of Congo red alone, obtained in the same conditions. Bars denote 1000 Å.

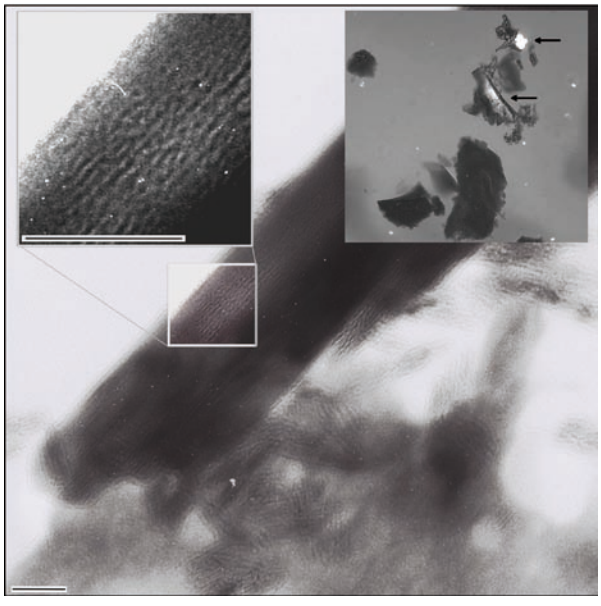

Figure 8. The part of the immunoglobulin L chain λ - Congo red aggregate of crystal-like character, as seen under an electron microscope. Insets – upper left: the enlarged fragment, upper right: the pieces of aggregate with portions of crystal-like nature (arrows), seen using an optical microscope in polarized light. Bars denote 1000 Å.

or if they aggregate mainly upon removal of the scaffolding dye, the obtained deposit particles of the Congo red-immunoglobulin L chain λ complex were treated with sodium dithionite. The reaction was halted before complete reduction of Congo red was achieved to prevent the splitting of protein disulfides. These particles obtained following the dye reduction were used for X-ray diffraction studies, after extensive washing with an acetate buffer (0.05 M, pH 5.4) and then 50% ethanol. The lyophilized immunoglobulin L chain λ (the same protein used for complexation with Congo red and for the formation of the ordered deposits) was used as the control sample. Superimposed diffraction patterns of the sample and the control are shown in the Fig. 10. The diffraction pattern of the sample, which is shifted and sharper compared to that of the control one, with reflection at around 4.7 Å, likely derives from the ordered molecular structures formed by proteins initially assembled along the Congo red scaffolding framework [43,44].

Despite the fact that the mechanism of the sample formation is different than that of standard amyloids, its diffraction pattern is similar. This suggests that the described procedure may offer an alternative approach to studies on processes of amyloid formation.

The toxicity of amyloids seems to be related mainly to their ordered, yet not very uniform structures [45-49]. However, the mechanism leading to the formation of such aggregates from partly unfolded protein molecules still remains unclear, even though many efforts to elucidate it have been made [50-56]. Attempts to understand the properties of ordered protein materials have a technological aspect as well, which may be investigated independently [57]. The techniques that may be used to study the mechanism of the phenomenon are those that deal with *in vitro* formation of protein aggregates.

Supramolecular forms of Congo red and some related dyes are fibrillar in nature, and under suitable conditions they may be used as scaffolding frameworks for introducing the desired order to the aggregating proteins, and, perhaps, to molecules of other origin as well. To perform the scaffolding role, the Congo red thread-like micellar structures need to be compact and rigid. At low pH values (below 6) such a form may be obtained due to protonation of the amino group and the resulting decreased repulsion of amphoteric dye molecules. However, the formation of long compact dye particles may also be triggered at higher pH values, by the application of an electric field which affects the charge-carrying supramolecular dye particles. The adjusted electrophoretic conditions impose an orientation on the dye particles, making them even more suitable for the scaffolding role. The scaffolding matrix arranged in the electric field introduces the ordered structure to the complex after the attachment of protein molecules. Large assemblies of particles formed in this way precipitate as amyloid-like material. However, the manner in which the electric field induces dye self-assembly remains unclear. The explanation which assumes π electrons are delocalized, and in this way influence the fibrillar dye structures as nano-wires seems to be reasonable.

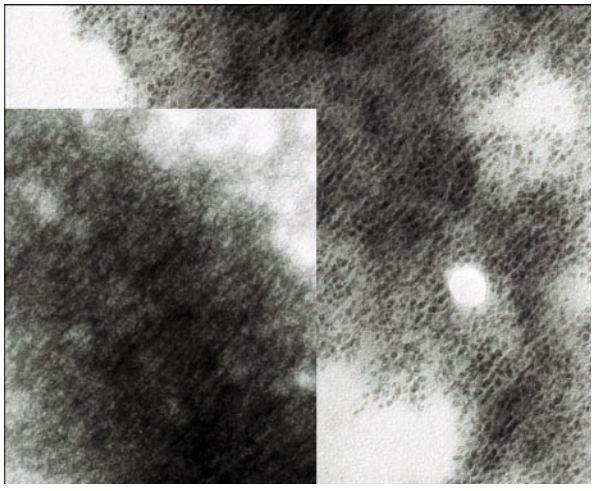

Figure 9. Electron microscope picture of the aggregated ferritin-Congo red complex (unstained). Inset - the same sample stained with uranyl acetate to reveal the Congo red scaffold (black streaks). Bars denote 1000 Å.

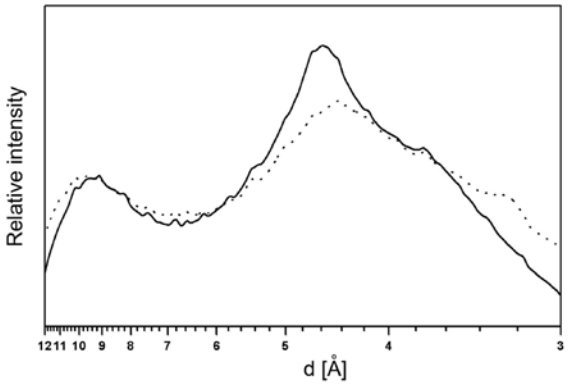

Figure 10. Relative intensity of X-ray pattern for amyloid obtained from immunoglobulin L chain λ (solid line) and the reference sample – lyophilized protein (dots) as a function of interplanar spacing d [Å].

As a result, the decreased π-π repulsion enhances self-association of Congo red and causes the equilibrium to shift toward the formation of longer and more rigid fibrillar structures. The possible influence of an external electric field on the delocalization of π electrons may be direct or mediated by changes in the self-assembly dependent charge of dye substituents - amino and sulfonic groups [58-63]. For neutral or alkaline solutions, *i.e.*, those with a pH above pK of the amino group, the negative charge of the sulfonate groups has a decisive influence on the charge of the dye. However, the sulfonate group's close proximity to the amino group in the self-assembled dye form may induce amino group protonation, even far from a pK value that would affect the net charge of the dye. On the other hand, the delocalization of π electrons connected with self-assembly competes with the protonation of the substituents, thus shifting the charge of the dye towards more negative values. This effect increases as the number of self-assembled molecules in supramolecular dye particles gets higher, which explains the differences in migration rates in agarose gel electrophoresis. In spite of the charge uniformity seen in electrophoresis, which is unfavorable for self-assembly, the dye particles, in response to the electric field, become more compact and rigid, indicating that the direct effect of the field on dye rigidity is dominant. The increased rigidity of dye particles makes their partition by proteins more difficult, and in consequence imposes the alteration of the mechanism of protein interaction with the dye. As may be expected, the high aggregation tendency of partly unfolded proteins enables the altered attachment of protein to the dye framework.

4. Conclusions

External electric fields can be a powerful factor that affects the self-assembly equilibrium of Congo red and favors the ordered supramolecular organization of this dye.

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