Biochemistry (Moscow), Vol. 70, No. 4, 2005, pp. 458-466. Translated from Biokhimiya, Vol. 70, No. 4, 2005, pp. 556-567.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-087, August 15, 2004.

Human Immunoglobulin Light Chains λ Form Amyloid Fibrils and Granular Aggregates in Solution

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Received April 2, 2004
Revision received May 24, 2004

Abstract—Myeloma nephropathy is a disorder characterized by deposition of monoclonal immunoglobulin light chains in the kidneys. The chains deposited form either amyloid fibrils or granular (amorphous) aggregates. Distinct molecular mechanisms leading to the formation of different aggregate types in kidney of patients with multiple myeloma are poorly understood. Here we describe the self-association kinetics of human monoclonal immunoglobulin light chains λ (GRY) isolated from urine of a patient with multiple myeloma. Under physiological conditions, the isolated light chain exists predominantly in a form of covalent dimer with apparent molecular mass of 50.1 kD. Spectral probe binding, analytical gel filtration, Western blot analysis, and electron microscopy indicate that GRY dimer aggregation occurs via two different pathways producing either amyloid fibrils or amorphous aggregates depending on microenvironment. Incubation of GRY (25 μM) for 4-14 days at 37°C in phosphate buffered saline (PBS), pH 7.0, or in PBS containing urea (0.8 M), pH 6.5, leads to amyloid fibril formation. Under electron microscopy, the fibrils show unbranched thread-like structures, ~ 60-80 × 1000 Å in size, which can bind thioflavin T and Congo Red. GRY maintained in acetate buffer, pH 3.5, forms granular aggregates. The structure of GRY oligomers formed during the early stage of amyloid fibril formation (1-4 days) has been examined by means of protein cross-linking with homobifunctional reagents. These oligomers are predominantly trimers and tetramers.

Key words: immunoglobulin light chains, amyloidosis, protein aggregation, multiple myeloma

Aggregation of soluble proteins followed by a formation of insoluble fibrils underlies a progression of some hereditary and acquired human disorders [1]. The most significant are Alzheimer's disease, diabetes type II, hereditary neuropathies, and primary systemic amyloidosis. Their general mechanism involves a deposition of regular cross-β fibrils (amyloid) in tissues whose structure is independent of the precursor protein type [2].

Kidney amyloidosis in multiple myeloma is one of these kinds of disorders [3]. This complication develops in 10-15% patients suffering from multiple myeloma and is the principal cause of death one to one and a half years after manifestation. Multiple myeloma is a malignant B-cellular dyscrasia accompanied by unrestricted synthesis of monoclonal immunoglobulins. The monoclonal immunoglobulin production results in accumulation of free immunoglobulin light chains in the blood of patients. Some portion of those light chains is not retained in the body, but excreted by the kidney in urine. Others, due to their thermodynamic instability, form insoluble aggregates in the kidney.

Different morphological types of light chain deposition can be observed in the kidneys of patients. Regular cross-β fibril formation leads to the development of renal AL-amyloidosis. It is a severe complication of multiple myeloma, which is insusceptible of present-day medication and characterized by fast progression and bad clinical prognosis. The second type of deposition, granular

Abbreviations: DSS) suberic acid *bis*(N-hydroxysuccinimide ester); BS^3) suberic acid *bis*(3-sulfo-N-hydroxysuccinimide ester); EDC) N-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PBS) phosphate buffered saline.

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0006-2979/05/7004-0458

amorphous aggregates, results in conditionally benign pathological process [3].

The studies on deposit morphology, primary structure of amyloidogenous light chains and their V_L domains, and folding of those proteins have not succeeded in unambiguous disclosure of a factor determining the self-association pathway of light-chains and the type (amyloid fibrils or granular aggregates) of their deposition in the human body [4]. Modern studies on light chain folding and association are intended to solve this problem.

Here we have studied the self-association kinetics of dimer of the human (GRY) immunoglobulin light chain λ isolated from the kidney of a patient with multiple myeloma. We have proved that this GRY protein can form two alternative types of associates, namely amyloid fibrils and granular aggregates. We have also found that the type of light-chain associates formed *in vitro* is determined by microenvironmental conditions. We have studied the structure of oligomers formed at the early stages of amyloid fibril production in solution of GRY protein.

MATERIALS AND METHODS

Chemicals. Tris, veronal, sodium phosphates, NaCl, $(NH_4)_2SO_4$, EDTA, polyethylene glycol 40,000, acrylamide, methylene-*bis*-acrylamide, ammonium persulfate, TEMED, SDS, glycine, and agarose were purchased from Sigma (USA). All of the acids used and NaOH (manufactured in Russia) were of chemical purity grade. The chromatographic sorbents Sephacryl S100 HR, DEAE-Sephacel, Blue-Sepharose 4B, Protein A-Sepharose 4B, and CNBr-Sepharose 4B were purchased from Amersham (USA).

Patient. A 64-year-old woman (GRY) was fist examined in the Department of Clinical Hematology, Moscow Regional Scientific Research Clinical Institute, in August, 2002. The diagnosis was manifested multiple myeloma with IgG-λ gammopathy, iliac osteoplasmocytoma, and chronic renal failure at stage I or II. Bone marrow aspirates revealed 5.4% of plasma cells. The concentration of Bence Jones protein in urine was 1.4 g/liter. The diagnosis of myeloma nephropathy had no morphological support since histological examination of the kidneys was not performed.

Isolation of GRY immunoglobulin light chains. The light chain of GRY immunoglobulins was isolated from a day's portion of urine (0.8 liter) of the patient with multiple myeloma. Urinary proteins were separated with 70% ammonium sulfate followed by ion-exchange chromatography on DEAE-Sephacel in 10 mM Na_2HPO_4, pH 8.1; elution with linear 0-300 mM gradient of NaCl. The fraction containing the light chain of immunoglobulin was additionally purified by means of adsorption on Blue-Sepharose 4B and Protein A-Sepharose 4B. The final purification step was gel filtration on Sephacryl S100 HR. The purified GRY protein (9.4 mg/ml) was maintained in 20 mM Na_2HPO_4, pH 7.4, containing 150 mM NaCl and 1 mM NaN_3 and stored at 4°C in sterile glass vials for no more than 48 h before experiment.

The purified GRY protein concentration was determined from absorption at 280 nm assuming A_{280} of 1% solution to be 12.5.

Protein homogeneity was assessed from SDS-PAGE by the Laemmli method [5] in 5-25% polyacrylamide gel. Both the protein purity and the isotype of light chain were determined by electrophoresis according to the method of Grabar and Williams in 1% agarose gel using antiserum against human plasma proteins (ICN, USA) and monospecific antisera against human immunoglobulin light chain types (Sigma). The purity of obtained specimen was no less than 95%.

Formation of amyloid fibrils and granular aggregates in GRY protein solution. Regular amyloid fibril or granular aggregate formation in GRY protein solution was achieved according to the previously proposed general approach [6].

The GRY protein was dialyzed for 48 h at 4°C against either 25 mM Na_2HPO_4, pH 7.0, containing 0.1 M NaCl (PBS), or 25 mM Na_2HPO_4, pH 6.5, containing 0.1 M NaCl and 0.8 M urea (PBS-urea), or 25 mM sodium acetate, pH 3.5, containing 0.1 M NaCl and filtered through a 0.22 μm Millipore filter (USA). The GRY protein solution (1.2 mg/ml, 25 μM, 0.75 ml) in corresponding buffer was incubated in borosilicate glass vials (12 × 75 mm) (Kimble, USA) for 14 days at 37°C. Solutions were stirred with a polypropylene rod (3 × 3 mm) on an orbital shaker at 450 rpm. The protein self-association was monitored each 24 h in 50 μl samples by the following biophysical methods.

Light scattering. The scattered light intensity (at a fixed angle of 90°) was detected in the 1.0 μM GRY protein solution on an MPF-4B fluorimeter (Perkin-Elmer, Japan) at equal excitation and emission wavelengths of 450 nm.

Thioflavin T binding. GRY protein solution (40 μl, 25 μM) was added into 2.0 ml of 100 mM Na_2HPO_4, pH 7.5, containing 100 mM NaCl and 18 μM of thioflavin T. The fluorescence was measured in the solution at 485 nm (excitation wavelength 450 nm) on the MPF-4B fluorimeter. The fluorescence intensity of the control specimen (18 μM thioflavin T only) was subtracted from the measured value.

Congo Red binding. The GRY protein solution (100 μl, 25 μM) was added to 200 μl of 100 mM Na_2HPO_4, pH 7.5, containing 100 mM NaCl and 20 μM Congo Red. After incubation for 1 h at room temperature, the differential absorption spectrum was recorded in the range 400-700 nm (1.0 cm quartz cell) on a Hitachi 2000M spectrophotometer (Japan). The reference cell contained 20 μM Congo Red only.

Electrophoresis. Electrophoresis of GRY protein was performed in 5-25% polyacrylamide gel under non-reducing conditions maintaining integrity of the protein disulfide bonds. No substances reducing disulfide bonds were used for both the sample and resolving gel preparation. Sodium dodecyl sulfate concentration in both electrode buffer and resolving gel was 0.1%. Denaturing SDS-PAGE by the Laemmli method [5] was performed in 5-25% polyacrylamide gel in presence of 40 mM 2-mercaptoethanol. Gels were then stained with Coomassie R250 (Sigma). Semi-dry protein electrotransfer onto a nitrocellulose membrane (Bio-Rad, USA) was performed in the buffer system described by Towbin et al. [7]. Immunochemical staining of the GRY protein adsorbed on the nitrocellulose membrane was performed using a monoclonal antibody raised against a linear epitope of free human λ-chain C_L domain. Immunochemical properties of anti-C_L (λ) mAB, which is produced by the hybridoma 5LA/3D12, were previously described [1]. Membrane with bound anti-C_L (λ) mAB was developed with rabbit polyclonal anti-mouse IgG-horseradish peroxidase conjugate.

Covalent cross-linking of the GRY protein with homobifunctional reagents. Suberic acid *bis*(N-hydroxysuccinimide ester) (DSS) and suberic acid *bis*(3-sulfo-N-hydroxysuccinimide ester) (BS^3) (Sigma) are homobifunctional reagents interacting with protein NH_2-groups (pH 6-8) and linking the protein molecules covalently via a CH_2-spacer eight carbon atoms in length. DSS (5 μl, 10 mM solution in dimethylsulfoxide) or BS^3 (4 μl, 5 mM solution in 20 mM Na_2HPO_4, pH 7.2) was added to 20 μl of 25 μM GRY protein dissolved in either PBS or PBS-urea. The mixture was agitated for 1 h at 24°C.

N-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is a cross-linking reagent interacting with protein COOH-groups (pH 6-8). EDC (4 μl, 10 mM solution in 15 mM Na_2HPO_4, pH 6.5) was added to 20 μl of 25 μM GRY protein dissolved in either PBS or PBS-urea. The mixture was agitated for 1 h at room temperature. Reaction was terminated with 62 mM Tris-HCl buffer, pH 8.0, containing 40 mM 2-mercaptoethanol, 2% SDS, and 10% glycerol. The cross-linked GRY specimens were separated in 5-25% acrylamide gel by the Laemmli method and analyzed by Western-blotting as described above.

Analytical gel filtration. Analytical gel filtration was performed using an FPLC system (Pharmacia, Sweden), profiles of protein elution being recorded at 280 nm. A HiPrep® Sephacryl S100 HR 16/60 chromatography column was used. GRY protein solution (25 μM, sample volume 50 μl) was applied on the column equilibrated with PBS, PBS-urea, or acetate buffer at the rate of protein elution of 0.5 ml/min at 24°C.

The apparent molecular weight of the protein was determined from the calibration plot of the elution volume versus molecular weight of protein standards. The calibration curve was drawn using the following standard proteins with known molecular weights: BSA dimer (132 kD) and monomer (66 kD), ovalbumin dimer (90 kD) and monomer (45 kD), chymotrypsinogen A (25 kD), and RNase (19.7 kD) (Pharmacia).

Analytical ultracentrifugation. The molecular weight of GRY immunoglobulin light chain was determined by analytical ultracentrifugation in 25 mM Na_2HPO_4, pH 7.0, containing 100 mM NaCl. Preparative gel filtration of GRY protein on Sephacryl S100 HR in corresponding buffer solution preceded the experiments on ultracentrifugation. Sedimentation equilibrium experiments were performed using an MOM 3170 analytical ultracentrifuge (Hungarian Optical Works, Hungary) at 21,340 rpm (±10 rpm) and constant temperature of 20°C (±0.2°C). The GRY protein concentration in the cell was 0.3 mg/ml in experiments on low-speed ultracentrifugation. Sedimentation equilibrium was essentially reached for 14-16 h in the sedimentation equilibrium experiments. Protein distribution along the centrifugation tube was monitored using a Rayleigh optical interference system. Mean (z-mean) molecular weight M_z of GRY protein in the sedimentation equilibrium experiments was calculated by the method of Van Holde and Baldwin [8]. The experiments on sedimentation velocity experiments were performed using a Beckman Spinco model E ultracentrifuge (USA) at 23,580 ± 10 rpm and constant temperature of 20 ± 0.2°C. The GRY protein concentration was 2-10 mg/ml. Mean (w-mean) molecular weight M_w of GRY protein was calculated by the method of Yphantis from the data of sedimentation velocity experiments [8].

Electron microscopy. The GRY protein solution was incubated in PBS or PBS-urea for 7 days or in acetate buffer for 1 day followed by fourfold dilution with corresponding buffer (final protein concentration 0.30-0.35 mg/ml). Protein solution was applied on carbon-coated grids and negatively stained with 1.8% solution of phosphotungstic acid (pH 7.0) or 1.0% uranyl acetate (pH 3.5). The samples were examined by a JEM 100C electron microscope (JEOL, Japan) at accelerating voltage of 80 kV.

RESULTS

Molecular forms of GRY immunoglobulin light chains initially present in solution. A solution of human immunoglobulin light chains is a heterogeneous system. The oligomerization of light chain monomers into dimers and tetramers as well as formation of complexes with distinct V_L and C_L domains results from noncovalent association and thiol-disulfide exchange in the solution [9, 10]. The equilibrium of the process is shifted to one of these molecular forms of light chain depending on the protein concentration and microenvironment. This is important in the study of protein self-association *in vitro* to charac-

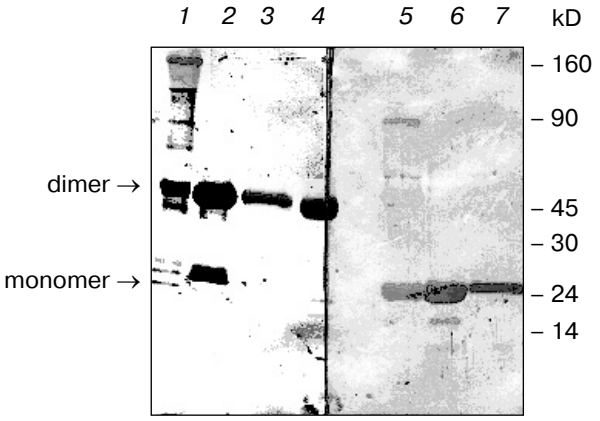

Fig. 1. Western-blot analysis of GRY protein. Proteins were separated by the Laemmli method in 5-25% polyacrylamide gel in the absence (lanes *1-4*) or presence of 40 mM 2-mercaptoethanol (lanes *5-7*) and transferred onto a nitrocellulose membrane. The membrane was incubated with monoclonal antibody against the linear epitope of C_L-domain of free human immunoglobulin light chain λ. Lanes: *1*, *5*) serum of the patient; *2*, *6*) urine of the patient; *3*, *7*) purified GRY light chain; *4*) standard covalent dimer of human immunoglobulin light chain. Arrows indicate the GRY molecular forms. Molecular weights of protein standards (kD) are given on the right.

terize a molecular form of immunoglobulin light chain, which serves as a "starting point" in the process of fibrilogenesis and protein aggregation in a solution. To solve the problem we determined the molecular weight of GRY light chain in 25 mM Na_2HPO_4, pH 7.0, containing 0.1 M NaCl using three independent biophysical approaches.

Electrophoresis of GRY protein in the absence of agents reducing disulfide bonds demonstrated that in solution the purified protein exists in a form of covalent dimer with molecular weight of ~50 kD (Fig. 1). Formation of GRY dimer results from the formation of disulfide bond between SH-groups of cysteines on C_L-domains of immunoglobulin light chain. 2-Mercaptoethanol (40 mM) added to the GRY protein results in complete transition of GRY to monomer with molecular weight of ~25 kD. Monomeric light chain as a stable form of the protein was only revealed by Western-blot analysis in the patient's blood and urine samples, in which it comprises 30-35% of total light chains (Fig. 1).

Figure 2 demonstrates the elution profiles of purified GRY protein (1.2-9.4 mg/ml) resulting from analytical gel filtration on a HiPrep® Sephacryl S100 HR 16/60 column in PBS, pH 7.0. From the data presented, the position and profile of the GRY elution peak depend on the protein concentration in the column.

This kind of dependence is indicative of an equilibrium between kinetically stable protein forms with different molecular masses existing in the GRY protein solution. As shown previously, a reversible noncovalent association of protein monomers to dimers and tetramers occurs in a solution of human immunoglobulin light chains [9, 10]. Analysis of these mixtures composed of reversibly interacting components by analytical gel filtration is rather complicated. The apparent elution volume depends on equilibrium constant of the association, concentration of light chains in the solution (column), column length, and properties of the medium [9, 10]. Apparent *w*-mean molecular weight of distinct molecular forms of immunoglobulin light chains therefore cannot be determined directly from the linear dependence of molecular mass on the elution volume of the corresponding protein form. The complete concentration dependence should be analyzed. GRY protein taken at the concentration of 1.2 mg/ml was eluted in PBS solution as an asymmetric peak with apparent molecular weight of 56 ± 3 kD (Fig. 2). This value is the same as the estimated *z*-mean molecular weight of GRY protein (56.3 kD), which was calculated from the data of sedimentation equilibrium experiments (Fig. 3a). Note that both M_z value and apparent molecular weight determined from analytical gel filtration are biased estimations sensitive to the contribution of high-molecular-weight components of non-homogeneous solution of immunoglobulin light chains. Exact evaluation of *w*-mean molecular weight M_w for GRY protein (50.1 kD) was derived from the data of sedimentation velocity experiments (Fig. 3b). It agrees with molecular weight of immunoglobulin light chain dimer.

Amyloid fibril formation in a solution of GRY immunoglobulin light chain. In the course of self-association of immunoglobulin light chain in solution, molecular complexes are formed with the particular quaternary structure of regular cross-β fibrils of amyloid.

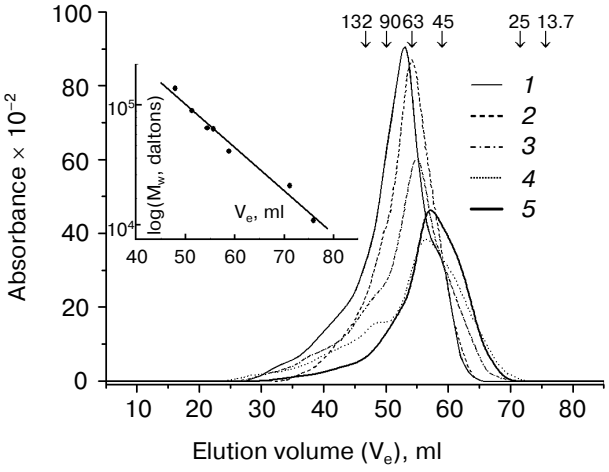

Fig. 2. Analytical gel filtration of GRY protein on a HiPrep® Sephacryl S100 HR 16/60 column in 25 mM Na_2HPO_4, pH 7.0, containing 0.1 M NaCl. Elution profiles are given for GRY protein concentrations of 9.4 (*1*), 4.7 (*2*), 3.1 (*3*), 2.4 (*4*), and 1.2 (*5*) mg/ml. Arrows indicate the elution volumes of protein standards. The calibration plot of elution volume (V_e) versus molecular weight (M_w) of protein standards is given in the frame.

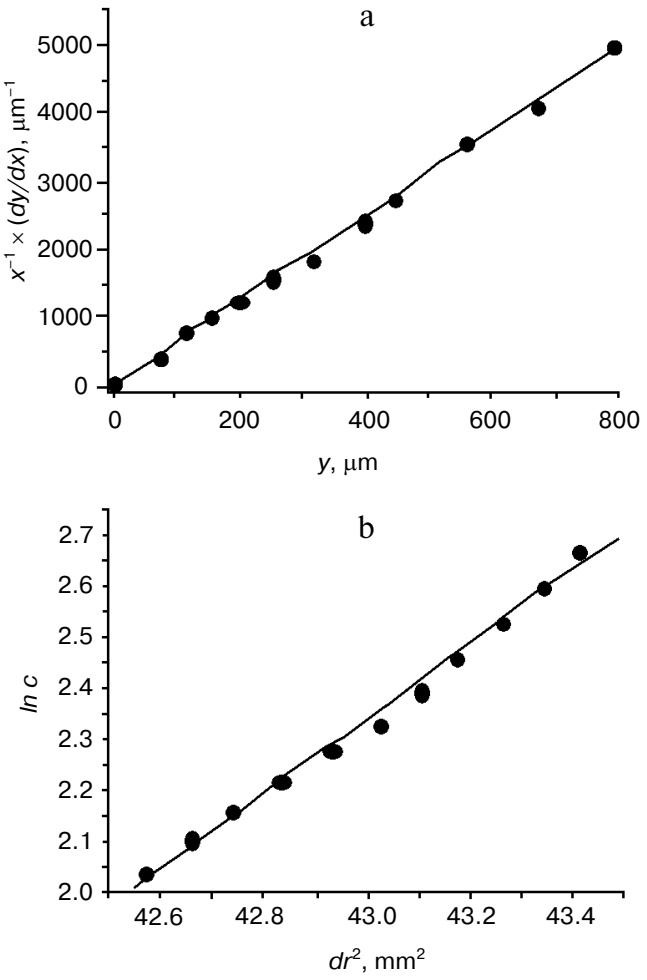

Fig. 3. Determination of the molecular weight of GRY protein in solution by analytical ultracentrifugation. a) Sedimentation equilibrium experiments of Van Holde and Baldwin: dependence of vertical shift of interference band (*dy/dx*) on its coordinate (*y*). b) Sedimentation velocity experiments of Yphantis: dependence of GRY sedimentation band shift (*dr*) on protein concentration (*ln c*) in the ultracentrifuge cell.

Three various buffers simulating the native physical and chemical conditions in different compartments of the kidney were used for GRY amyloid fibril formation [11, 12]. PBS, pH 7.0, corresponds by its composition to human blood plasma and primary filtrate of urine within a capsule of the renal glomerulus. PBS-urea (0.8 M), pH 6.5, simulates the microenvironment corresponding to the distal tubule of nephron and interstitial renal medulla. The concentration of urea in the distal tubule and interstitial tissue depends on nitrogen metabolism and varies in the range of 0.4-1.5 M [12]. The mean urea concentration from this range was used in the incubation buffer for GRY protein. It is worth noting that this concentration is far out of the range of denaturation transition (3-4 M urea) for light chain dimers of human immunoglobulins [13]. At last, acetate buffer, pH 3.5, corresponds to the local acidic medium characteristic of the proximal tubule and the loop of Henle [12].

Kinetics of amyloid fibril formation in GRY protein solutions *in vitro* was studied by spectral methods (UV fluorescence spectroscopy and light scattering), as well as by direct visualization of immunoglobulin light chain complexes (electron microscopy) according to the general approaches published in [4, 6].

Light scattering. Light scattering in a region far from the absorption band at 450 nm can be used to follow the kinetics of accumulation of high-molecular-weight aggregates whose hydrodynamic radius is comparable with the wavelength of the scanning light beam. The dependence of the scattered light intensity on incubation time in GRY protein solution is given in the Fig. 4. The data presented show that early signs of self-aggregation become only detectable after four-day incubation in PBS or PBS-urea.

Thioflavin T binding. Kinetics of amyloid fibril formation in GRY protein solutions was studied using the specific fluorescence probe thioflavin T (Fig. 4). It was shown previously that the intensity of thioflavin T fluorescence increases with binding with extended β-sheets formed by immunoglobulin light chain monomers or other amyloidogenous proteins on their aligning along the axis of the amyloid fibril [4, 6]. The data presented indicate that the formation of amyloid fibrils in GRY solutions in PBS and PBS with urea begins after four days of incubation. Completion of amyloid fibril formation in PBS and PBS-urea requires no less than 14 days (Fig. 4).

Congo Red binding. Congo Red is a specific (absorption) probe by means of which the formation of regular amyloid fibrils has been studied [4, 6]. The absorption peak of the probe in a buffer solution at pH 6-8 without protein is about 490 nm. Binding of Congo Red with amyloid fibrils results in a shift of absorption maximum to ~540 nm, which is simultaneously accompanied by the increase of the probe absorption in this band. Absorption ratio A_{470}/A_{540} is a characteristic parameter whose increase to more than 1.0 is evidence for amyloid fibril formation in a protein solution [4, 6]. The GRY protein incubated in PBS or PBS-urea shifts the absorption maximum of Congo Red and leads to 15-25% increase in absorption at 540 nm. From the data on Congo Red binding, the kinetics of amyloid fibril formation in GRY protein solution is similar to that registered upon the binding of thioflavin T (Fig. 4).

Electron microscopy. The morphology of amyloid fibrils formed by GRY protein dissolved in PBS or PBS-urea was studied by transmission electron microscopy. Figure 5a shows non-branched fibrils 60-80 Å in diameter and of varied length (850-1000 Å) formed in GRY solution in PBS after incubation for 7 days. The same pattern of GRY protein fibrils was observed after incubation in PBS-urea (0.8 M) for 7 days (Fig. 5b).

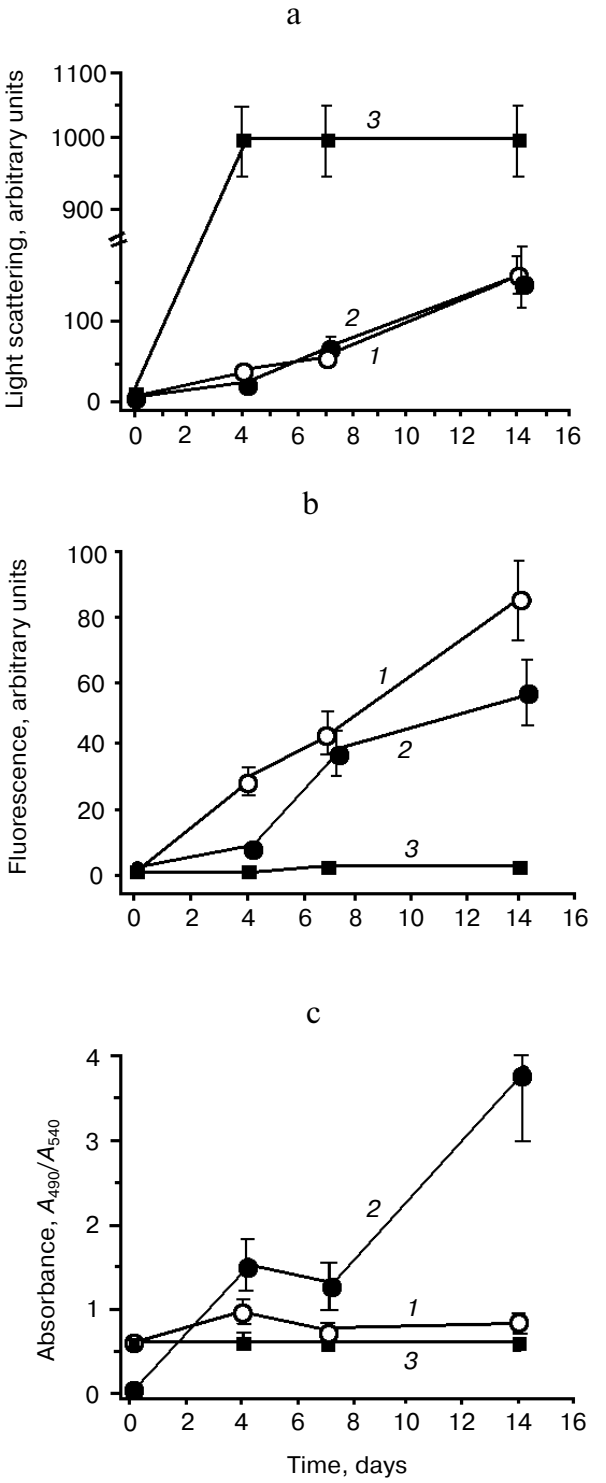

Fig. 4. Kinetics of amyloid fibril formation in GRY protein solutions. The plots show a progression of scattered light intensity at 450 nm (a), thioflavin T fluorescence at 485 nm (b) and absorption of Congo Red at 490 and 540 nm (c) with time. GRY protein (25 μM) was incubated at 37°C in PBS, pH 7.0 (*1*), PBS-urea (0.8 M), pH 6.5 (*2*), and acetate buffer, pH 3.5 (*3*). Protein aliquots were sampled each 24 h and analyzed as described in "Materials and Methods".

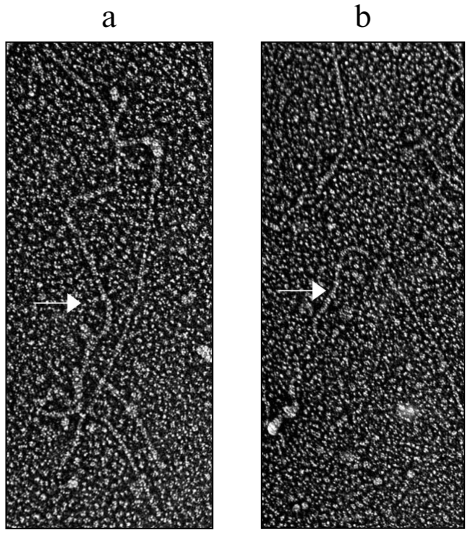

Fig. 5. Electron microscopy of GRY fibrils in solution. GRY protein (25 μM) was incubated in PBS, pH 7.0 (a), or in PBS-urea (0.8 M), pH 6.5 (b), for 7 days at 37°C with agitation and examined by the electron microscope. Samples were negatively stained with phosphotungstic acid. Arrows indicate distinct fibrils. The arrow length is ~140 Å.

Formation of granular aggregates in a solution of GRY immunoglobulin light chain. When incubated in acetate buffer, pH 3.5, the GRY protein forms granular aggregates in one day. This is evident from the rapid formation of high-molecular-weight aggregates as judged from the light scattering, and from the lack of Congo Red and thioflavin T (probes specific for regular cross-β structure) binding (Fig. 4). The morphology of aggregates formed in GRY protein solution at pH 3.5 is shown in the Fig. 6. They are granular protein aggregates with irregular quaternary structure.

Thus, the dataset gained using spectral methods and electron microscopy indicate that the human (GRY) immunoglobulin light chain (λ) dimer forms different associate types, namely regular amyloid fibrils and granular aggregates, depending on microenvironment. Amyloid fibril formation occurs in solutions of GRY protein in PBS, pH 7.0, and PBS-urea (0.8 M), pH 6.5, after 4 days of incubation, and the formation of amorphous aggregates occurs at pH 3.5 during the first day of incubation.

Formation of immunoglobulin light chain oligomers at early stages of amyloid fibril formation in GRY protein solution. Figure 7 shows the GRY protein elution profiles during analytical gel filtration at various incubation times of the protein in PBS solution, pH 7.0. The height of the elution peak corresponding to the dimer fraction, the main molecular form of GRY in solution, decreases with incubation time. The elution volume of GRY protein is moving toward the free volume of the chromatography

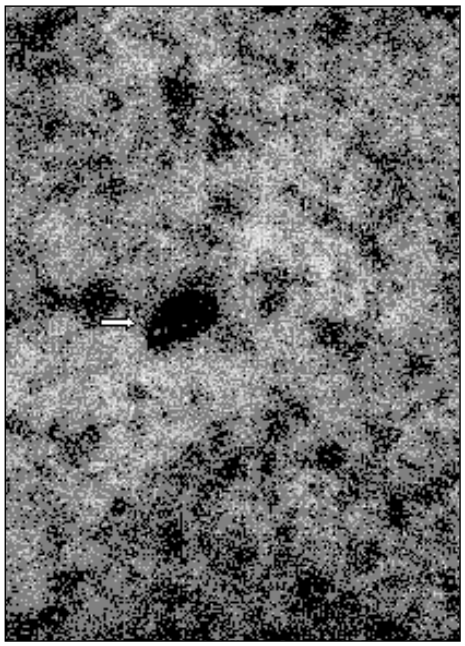

Fig. 6. Electron microscopy of GRY granular aggregates in solution. GRY protein (25 μM) was incubated under agitation in acetate buffer, pH 3.5, overnight at 37°C and examined by the electron microscope. Samples were stained with uranyl acetate. Arrows indicate individual aggregates. The arrow length is ~1000 Å.

column (V_0 ~ 41 ml) and corresponds to the free volume after 7 days of incubation in PBS. This is evidence for self-association of GRY protein and formation of higher-order oligomers at the expense of dimer, which was initially present in the protein solution.

Elution profiles of GRY protein incubated in PBS-urea (0.8 M), pH 6.5, correspond to those obtained for the of light chain dimer in PBS, pH 7.0 (elution profiles not shown).

Accumulation of "early" oligomers upon fibril formation in the GRY protein solution occurs after four days of incubation in PBS and PBS-urea. In fact, the elution volume of GRY protein after 4-day incubation (~52 ml) exceeds that of full-length fibril (~41 ml), but is smaller than the elution volume of native dimer of GRY light chain (~56 ml) (Fig. 7). Thus, the elution profile of GRY protein after 4-day incubation in PBS or PBS-urea reflects molecular-weight distribution of "earlier" oligomers upon fibril formation; however, it cannot be used for direct determination of *w*-mean molecular weights of individual protein oligomers.

To determine the molecular weights of earlier GRY oligomers through accumulation of which the formation of regular amyloid fibril occurs in PBS and PBS-urea solutions, the method of covalent cross-linking of protein oligomers with homobifunctional reagents was employed.

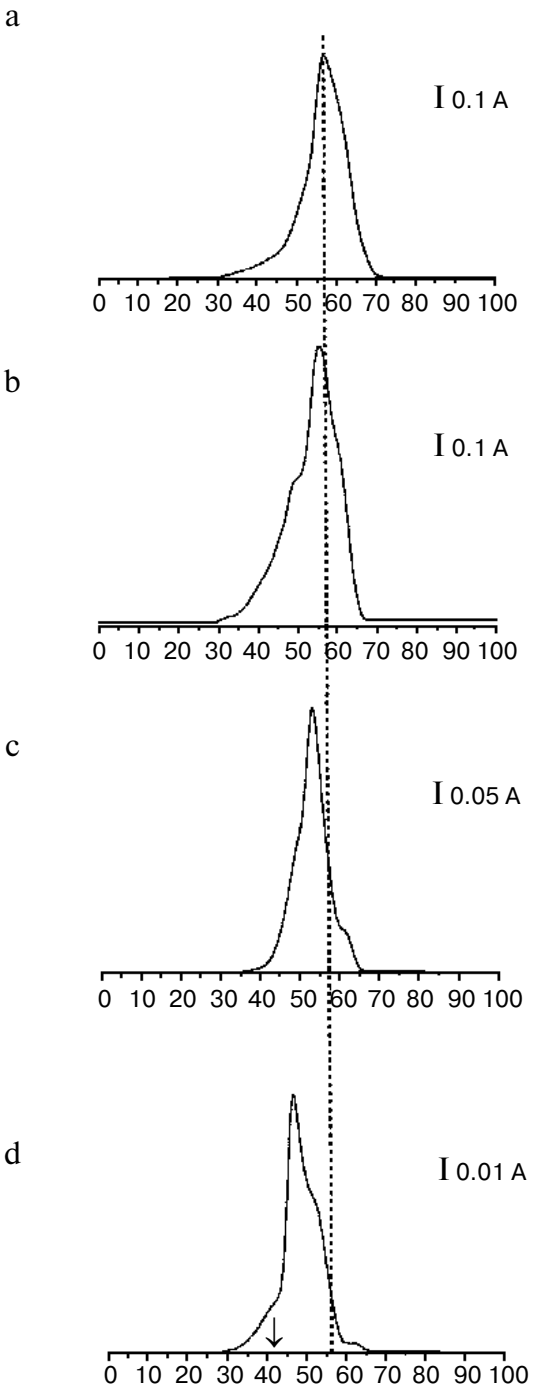

Fig. 7. Analytical gel filtration of GRY oligomers during fibril formation in solution. GRY protein (25 μM) was incubated with stirring in PBS, pH 7.0, at 37°C. Aliquots were sampled and subjected to chromatography on the HiPrep® Sephacryl S100 HR 16/60 column in 25 mM Na_2HPO_4, pH 7.0, containing 0.1 M NaCl. Elution profiles of GRY protein prior the incubation (a) and after 1 (b), 4 (c), and 7 (d) days are given. The arrow indicates elution volume for thyroglobulin (660 kD) corresponding to the free volume of the column. The elution volume of GRY dimer (50.1 kD) prior the incubation is shown by the dotted line. The dashed line (×*A*) indicates the absorption of the protein solution at 280 nm.

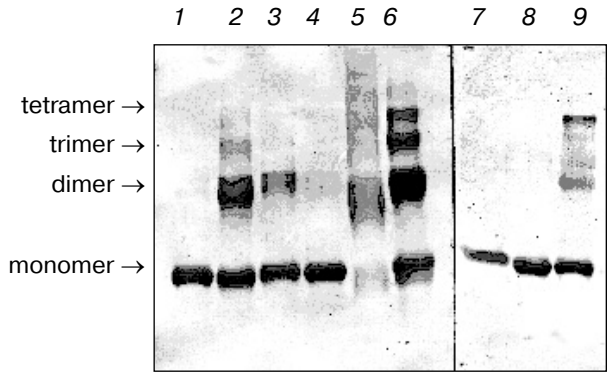

Fig. 8. Western-blot analysis of GRY oligomers formed at early stages of amyloid fibril formation. GRY protein (25 μM) was incubated under agitation in PBS, pH 7.0 (*1-3*), and PBS-urea (0.8 M), pH 6.5 (*4-6*), for 4 days at 37°C. Control samples: GRY protein (25 μM) in PBS, pH 7.0 (*7-9*), were incubated without agitation for four days at 4°C. Afterwards a covalent linking of GRY protein oligomers was performed in solutions using BS^3 (*1*, *4*, *7*), DSS (*2*, *5*, *8*), and EDC (*3*, *6*, *9*). After separation in polyacrylamide gel (5-25%) by the Laemmli method, the protein was transferred onto a nitrocellulose membrane and incubated with monoclonal antibody to linear epitope of C_L-domain of free human immunoglobulin light chain λ. Arrows indicate molecular forms of GRY.

Figure 8 shows the data on separation of "earlier" oligomers by Laemmli electrophoresis after 4-day incubation of GRY protein in PBS and PBS-urea and cross-linking of the resulting oligomers with DSS, BS^3, and EDC. Visualization of covalently linked GRY oligomers by Western-blot analysis was performed using monoclonal antibody to linear epitope of C_L-domain of free human immunoglobulin light chain λ [1].

According to the data presented, trimers (~75 kD) and tetramers (~105 kD) are the oligomeric GRY protein forms present in solutions at early stages of fibril formation together with dimer (~50 kD) and monomer (~25 kD) (Fig. 8). Trimers and tetramers of immunoglobulin light chain are absent in control solutions of GRY protein in PBS and PBS-urea after incubation without stirring for four days at 4°C (Fig. 8). The qualitative composition of cross-linked GRY oligomers is not the same for all homobifunctional reagents used. The discrepancy is associated with the yield of linked products and probably with their ability to react with anti-C_L (λ) monoclonal antibody.

Within the approach used, one cannot be sure that trimer and tetramer are the only GRY oligomers present in PBS and PBS-urea at early stages of fibril formation. Restrictions of Western-blot analysis in the study of "earlier" oligomers are associated with properties of the monoclonal antibody used, which recognizes linear epitopes of C_L-domains of free light chains. Some data indicate that C_L-domain in amyloid fibril undergoes limited proteolysis and possible steric shielding upon polymerization of the monomer [14]. For this reason, the monoclonal antibody used in our study can be "blind" to epitopes present in other, higher-molecular-order GRY oligomers formed at earlier stages of fibril formation.

DISCUSSION

Kidney deposition of immunoglobulin light chains complicates multiple myeloma progression and significantly aggravates clinical prognosis [3]. The study of kidney from patients with multiple myeloma by electron microscopy has demonstrated that immunoglobulin light chains form two deposition types differing by their morphology: amyloid fibrils and granular (amorphous) aggregates [3]. Purified immunoglobulin light chains isolated from patients with multiple myeloma form associates with similar morphology in solution *in vitro* [6].

The importance of the study on the structure of light chain depositions in tissues of patients with multiple myeloma is dictated by the fact that morphology of the light chain associate determines the clinical variant of kidney impairment in the patients and, hence, the prognosis of the disease [3]. *AL-Amyloidosis* characterized by deposition of amyloid fibrils in kidney, which are composed of full-length immunoglobulin light chains, their proteolytic derivatives, and individual V_L-domains, is a most inauspicious complication of multiple myeloma. Kidney deposition of amorphous (granular) aggregates of light chain (*LCDD*, *light chain deposition disease*) is not so dangerous and is characterized by more auspicious clinical prognosis [3]. The molecular mechanisms determining the type of immunoglobulin light chain associate *in vivo* are still unclear. Examination of kidney from patients with multiple myeloma leads to the conclusion of uniform deposition of light chain in the body of the individual patient. A possibility of simultaneous deposition of light chain by the pathways of amyloid fibrils and granular aggregates has not been proven.

We have demonstrated the effect of amyloid cross-β fibril and granular aggregate formation by the example of human immunoglobulin light chain (λ) dimer. GRY protein was isolated from urine of a patient with multiple myeloma. The data on binding of thioflavin T and Congo Red and electron microscopy are evidence for amyloid fibril formation in GRY protein dissolved in PBS, pH 7.0, and PBS-urea (0.8 M), pH 6.5, after 4-day incubation. In contrast, incubation of GRY protein in acidic buffer, pH 3.5, results in formation of granular aggregates with irregular quaternary structure. Thus, various in nature, but relatively mild denaturing conditions, such as stirring in 0.8 M urea solution, pH 6.5, and in acetate buffer, pH 3.5, result apparently in the formation of divergent molecular structures (amyloid fibrils and granular aggregates) by the same protein.

The formation of two different types of associates from light chain alone was demonstrated earlier on the

model of recombinant V_L-domain of SMA amyloidogenous light chain [15]. In this case, two different conformers of SMA V_L-domain, which represent partially structured non-native protein forms, are the precursors of amyloid fibrils and amorphous aggregates. After their accumulation in a solution, oligomerization of SMA V_L-domain only develops via one of two pathways: either through the formation of amyloid fibrils or through the formation of granular aggregates. According to the model, the microenvironmental conditions favoring the settlement of one or the other non-native conformer of SMA V_L-domain determine the type of the protein self-association *in vitro*.

An alternative view on the process of formation of two associate types by individual light chain is that somatic mutations in the genes encoding light chains lead to appearance of amino acids in the V_L-domain sequence destabilizing polar and hydrophobic interactions inside the domains [16, 17]. Realignment of this interaction system results in the oligomerization of native (quasi-native) V_L-domains and subsequent building of amyloid fibrils and amorphous aggregates on the basis of these oligomers. Thus, according to this conception, the character and position of amino acid residues arising in the process of mutagenesis in genes encoding immunoglobulin light chains are the driving force for the process of self-association of immunoglobulin light chains [16, 17].

Which of the two mechanisms is realized under self-association of full-length immunoglobulin light chains and their dimers *in vivo* remains unclear. Note that it is full-length immunoglobulin light chain and its dimer, but not individual V_L-domains, that are circulating in blood and filtered by kidneys into urine in patients with multiple myeloma, thus being molecule-precursors of amyloid deposition in tissues [3, 6, 11]. Therefore, the data obtained from experiments with recombinant V_L-domains ("half" or "quarter" of immunoglobulin light chain) are of limited value.

Our results suggest that the process of amyloid fibril formation in GRY solution develops through the stage of accumulation of "earlier" protein oligomers—trimers and tetramers of immunoglobulin light chains. The formation of "earlier" light chain oligomers is a part of nucleation stage followed by the proper filament growth and amyloid fibril formation [6]. The formation of "earlier" oligomers (dimers, tetramers, and octamers) was demonstrated earlier on the model of fibril formation of *recombinant V_L-domains* of amyloidogenous immunoglobulin light chains [18, 19]. However, it is agreed that the formation of trimers, tetramers, or oligomers of higher order in the process of amyloid fibril formation from *full-length immunoglobulin light chains* is not proved, despite the fact that these structure types were detected in blood and urine of patients with multiple myeloma [11, 20, 21]. In this connection, there is no answer to the question of the place of self-association of noncovalent immunoglobulin light chain trimers in the hierarchy of early events. Accumulation of GRY light chain trimers has been demonstrated upon formation of amyloid fibrils in PBS and PBS-urea. From our point of view, the light chain trimer is not an individual "earlier" oligomer of the nucleation process. The fibril growth *in vitro* is a complex process including concomitant secondary reactions of oligomer fragmentation, their lateral growth, and heterogeneous association. The accumulation of the immunoglobulin light chain trimer in GRY protein solution upon the fibril formation is possibly a result of these concomitant reactions.

This study was supported by the Russian Foundation for Basic Research (grant Nos. 04-4-97254 and 04-04-97321).

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