Signature: Med Sci Monit, 2003; 9(4): BR185-193
PMID: 12709666

Received: 2002.06.07
Accepted: 2003.01.03
Published: 2003.04.23

The structural abnormality of myeloma immunoglobulins tested by Congo red binding

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Paweł Spólnik[1], Barbara Piekarska[1], Barbara Stopa[1], Leszek Konieczny[1], Grzegorz Zemanek[1], Janina Rybarska[1], Marcin Król[2], Mateusz Nowak[2], Irena Roterman[2]

[1] Institute of Medical Biochemistry, Collegium Medicum, Jagiellonian University, Cracow, Poland
[2] Department of Biostatistics and Medical Informatics, Collegium Medicum, Jagiellonian University, Cracow, Poland

Source of support: This study was supported by the Polish Scientific Research Commission (KBN), research grant no. 6 P05F012 20. Sera were obtained from the Department of Laboratory Diagnostics, Chair of Clinical Biochemistry, Collegium Medicum, Jagiellonian University.

Summary

Background: Frequently observed structural deviations of myeloma-derived immunoglobulins affect polypeptide chain packing and domain stability, enhancing their tendency to aggregate, with all the clinical consequences. Congo red complexation with myeloma immunoglobulins is proposed in this work as a general test to disclose the instability of these proteins. The large ribbon-like supramolecular ligands of Congo red form complexes with proteins by adhesion to β-conformation polypeptide chains, if allowed to make contact with their backbone interfaces. This can occur in the case of myeloma-derived immunoglobulins with deficient polypeptide chain packing.

Material/Methods: Specially adapted two-dimensional agarose electrophoresis of serum proteins, which allows the transient contact of Congo red and serum proteins during migration, was used to reveal the presence of protein components amenable to ligand penetration and binding. The combination of electrophoresis and Congo red binding to proteins permits the removal of loosely attached dye and evaluation of the effective complexation properties of the immunoglobulin fraction directly in the serum.

Results: Comparative studies of dye complexation with two L chains having different reactivities with Congo red confirmed that dye binding depended on protein instability in the conditions used. Myeloma proteins revealed different binding capabilities in the test used here.

Conclusions: The complexes formed by the supramolecular dye Congo red with myeloma immunoglobulins differ in stability. Those of high stability indicate the abnormal protein structure thought to produce clinical symptoms. This work proposes an easy technique to differentiate the stability of complexes.

key words: monoclonal gammopathies • Congo red • protein instability

Full-text PDF: http://www.MedSciMonit.com/pub/vol_9/no_4/2827.pdf
Word count: 3172
Tables: –
Figures: 7
References: 60

Author's address: Janina Rybarska PhD, ul. Kopernika 7, 31-034 Kraków, Poland, email: mbstylin@cyf-kr.edu.pl

BACKGROUND

The structural abnormality of myeloma immunoglobulin proteins can often be detected directly by their significantly altered molecular weight or unusual properties [1,2]. However, the majority of monoclonal proteins observed by clinical tests are at least apparently normal, although many of them actually carry barely noticeable structural faults which may lead to fatal consequences, arising basically from the increased tendency of their molecules to aggregate [3,4]. It is important to find a common general feature of myeloma protein that can help to predict its higher association propensity and can be used to develop simple clinical tests.

In approaching this problem we assumed that any structural deformation of the polypeptide chain or specific atypical changes in amino acid residues result in packing deterioration and decreased domain stability, compared to the corresponding normal native minimum-energy structure [5–8]. The less compact protein packing can in turn be registered as the molecule's increased susceptibility to penetration by various ligands, such as the often used fluorescent dye ANS (8-anilino-1-naphtalenesulfonic acid). Such an effect is actually observed upon protein melting, which is a non-native structural transformation involving the hydrophobic core [9–13].

Unfortunately, most structural alterations associated with commonly observed myeloma proteins seem to render accessible only rather peripheral protein regions of moderate hydrophobicity, thus with limited ability to attach nonspecific ligands. However, an exceptional type of binding permitting the detection of decreased packing stability is presented by Congo red dye and some related compounds with high self-assembling activity, which in water solutions form ribbon-like, supramolecular, polyanionic structures with specific interaction properties [14–16]. Oligo- or polymolecular assemblies derived from Congo red micelles form specific supramolecular dye ligands capable of adhering to structurally corresponding β-conformation polypeptide chains [17–19]. Because of the great extent of contact in complexation, plasticity, and in particular the significant exposure of the hydrophobic portion of the ribbon-like supramolecular ligand, Congo red binding has the character of a specific interaction, making the resulting stability of the complex much higher than predicted for single-molecule binding [19–22]. To reach the polypeptide chain backbone interface, a condition for proper complexation, the dye ligand must be allowed to penetrate the protein body. This is simple in clearly unstable proteins. For Congo red, however, it also seems possible in the case of at least transient local protein deformations in polypeptide chain packing [17,18,22].

While native, well-packed immunoglobulin domains may become accessible for dye penetration and binding at elevated temperature only, their structurally abnormal derivatives usually bind Congo red without heating. Thus Congo red would seem to be suitable as the sought-after probe of protein instability. The nature and high stability of the protein-dye complex renders structural deviations of myeloma proteins detectable even if standard clinical test results seem normal.

This work proposes a simple technique using Congo red to disclose evident and discreet instabilities of myeloma proteins, which may enhance the propensity to aggregate.

MATERIAL AND METHODS

The reagents used were the following:
- Congo red (Aldrich Chemical Co. Inc. Milwaukee, USA);
- Sandoglobulin-P (Sandoz Pharma Ltd, Switzerland);
- Bio-Gel P-6 (BIORAD Lab, California, USA);
- agarose SERVALYT PRECOTES 5–9 gels (SERVA, Germany);
- Sephacryl S-300 (PHARMACIA, Sweden);
- DPCC-trypsin (trypsin treated with diphenyl carbamyl chloride - devoid of trace chymotrypsin activity);
- PSMF (phenylmethylsulfonyl fluoride), purchased from SIGMA Chemical Co. (St. Louis, Missouri, USA).

All other reagents were of analytical grade:
- TCL silica gel 60 (Merck, Germany)
- Rabbit antibodies against human IgG (DAKO, A/S, Denmark).

Analysis of Congo red-IgG immunoglobulin complexes

Myeloma IgG was isolated by direct elution from the agarose gel after electrophoresis of the patient's serum. Normal polyclonal IgG was obtained by gel filtration of Sandoglobulin-P on a Sephacryl S-300 column protein concentration measured spectrophotometrically (A at 280 nm for 0.1% equals 1.4). Samples containing the myeloma or polyclonal IgG with a 60-fold molar excess of Congo red were incubated for 15 min at appropriate temperatures, then the dye-protein complexes were isolated by gel filtration on BioGel P6 columns. The dye-protein ratio in the complexes was estimated after developing chromatograms on TCL silica gel 60 plates in methanol-water solution (3:2). The Congo red was eluted from the bed and determined spectrophotometrically (A at 490 nm for 0.1% equals 46). The protein content was estimated by extracting and measuring the bromophenol blue used to stain it.

Formation and analysis of Lλ chain-dye complexes

Human immunoglobulin chain Lλ chain dimer proteins were isolated from the urine of patients with multiple myeloma by salting out with ammonium sulfate and subsequent gel filtration on a Sephacryl S-300 column. The concentration of L chains was estimated spectrophotometrically (A at 280 nm for 0.1% equals 1.2). Analysis by SDS PAGE (in reducing and nonreducing conditions) revealed that both the L chain dimers were linked by disulfide bonds. Samples containing the Lλ chain (5 mg/mL, in 0.1 M pH 7.0 phosphate buffer, 0.9% NaCl) and Congo red (20-fold molar excess to the Lλ monomer) were incubated at a given temperature for 15

min. The presence of dye-protein complexes was revealed by agarose gel electrophoresis (1% agarose, sodium barbiturate buffer pH 8.6). The gels were stained with bromophenol blue. The susceptibility of dye-protein complexes to proteolysis was analyzed by digestion with DPCC-treated trypsin, at 1:50 (w/w) enzyme: Lλ monomer ratio, in Tris-HCl buffer pH 8.0, 0.9%NaCl, for 20 min at 20°C. The reaction was blocked with PMSF.

Two-dimensional electrophoresis of serum proteins

The first run was performed by standard method (1% agarose, 0.05 M sodium barbiturate buffer pH 8.6). Before the second run a solution of Congo red (5 mg/mL) was applied to the gel, below and along the protein start line through a strip of net material for screen printing (180 mesh). After electrophoresis the gel was washed briefly (1 min) with water-methanol solution (7:3 v/v), then stabilized for 15 min in a mixture of saturated picric and acetic acids (5:1 v/v). The stabilized gel was washed with methanol until the Congo red spots turned back to red. The free dye was removed by blotting with wet filter paper, and hot air dried gel was stained with bromophenol blue dye.

Reversed electrophoresis of serum proteins

Electrophoresis was run in standard conditions, except that the gel and buffer contained soluble CM-cellulose (0.5%, Sigma, USA). In this system the migration of serum proteins is reversed. Fast migration of immunoglobulin proteins significantly facilitates their analysis. All steps preceding staining with amido black were performed as described above. Cytochrome c was chosen as the migration indicator.

Amino acid sequence analysis

Amino acid sequence analysis of the N-terminal parts of the Lλ chains was performed on a gas-phase sequencer (Model 491, Perkin Elmer-Applied Biosystems, Foster City, California, USA).

Carbohydrate detection

The carbohydrate component was determined using labeled lectins (DIG Glycan Diferentiation Kit – Roche Molecular Biochemicals, Germany).

Computer simulation

The computer simulation used the CHARMM program (Harvard University) for 1, 2, 4, 6, 8 and 9 ns molecular dynamics of the model peptide 1-21 aa N-terminal fragment as it appeared in 4BJL (PDB file) light chain λ dimer 1 in two forms: (1) the natural form, with Pro at position 13, and (2) mutationaly changed Pro13Leu, to reveal the proline's influence on the stability of the polypeptide structure.

Independently, molecular dynamics were simulated for two forms of light chain dimer: (1) the natural form as it appeared in PDB (4BJL), and (2) a polypeptide chain deprived of 1-21 amino acid residues to reveal the N-terminal fragment's role in the stability of the protein.

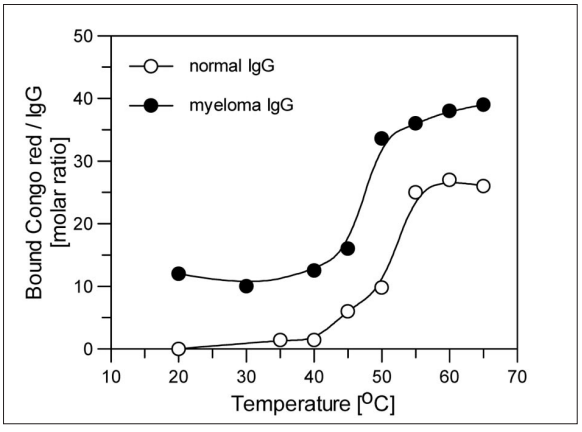


Figure 1. Differences in accessibility of normal and myeloma-derived IgG to Congo red binding, measured at increasing temperatures. Samples containing the studied proteins with a 60-fold molar excess of Congo red were incubated for 15 min at appropriate temperatures. The dye-protein complexes were isolated by gel filtration on BioGel P6 columns for analysis.

RESULTS

No β-structure able to act as a receptor for Congo red is available for complexation in native well-packed proteins. Appropriate susceptibility seems necessary for a protein to bind a large ligand composed of self-assembled dye molecules. Complexation is hence possible in unfolding conditions, although the constraints associated with antigen binding in immunoglobulins have also been found to generate sufficient structural alterations [15,17,18,23–25].

Myeloma immunoglobulins that form complexes with Congo red readily without heating may thus be suspected to have abnormal structure. The difference in Congo red binding between heated, normal and some randomly selected myeloma immunoglobulins is shown in Fig. 1.

Our research intended to verify the dependence of Congo red binding on protein instability, based in this work on two human myeloma-derived Lλ chains (called here An and Kok), having significantly different dye-binding capabilities. L chains were chosen for model studies as the simplest protein molecules still representing the structure of standard immunoglobulin polypeptide chain fold.

The experiments indicated that some weak, easily removable surface protein-dye interaction with no noticeable relation to protein stability usually occurs in the presence of dye excess. However, complexes that persist in electrophoretic conditions and that migrate as definite fractions – or similarly, complexes separated from the free dye by gel filtration – are formed only if the packing stability of the polypeptide chain is deficient, allowing the dye to penetrate and reach polypeptide backbone interfaces.

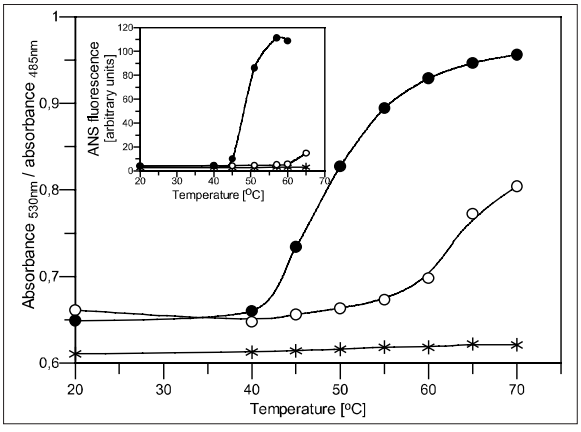


Figure 2. Complexation-derived shifts in the Congo red spectrum observed at different temperatures in samples containing L chain λ and the dye. (○) Congo red + Lλ(An), (●) Congo red + Lλ(Kok), (∗) Congo red (control). Insert: Fluorescence (λ exc=370 nm) effect found in a corresponding complexation experiment with ANS. Samples containing Lλ chains (1 mg/mL, in 0.1 M phosphate buffer pH 7.0, 0.9% NaCl) and ANS or Congo red (both at 4-fold molar excess to the Lλ monomer) were incubated at a given temperature for 15 min.

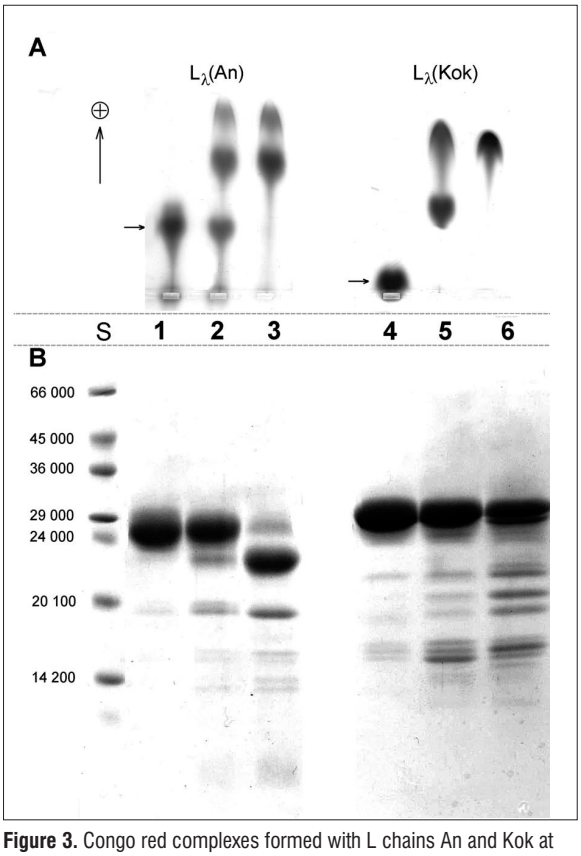


Figure 3. Congo red complexes formed with L chains An and Kok at 20°C and 45°C as seen in agarose electrophoresis (A), and also registered by facilitated proteolysis, revealed by SDS-PAGE (B). The spots in panel A represent the bromophenol blue-stained proteins (Congo red was removed from complexes before staining by reduction with sodium dithionite). Fractions migrating faster than uncomplexed L chains (indicated by arrows) represent the positions of Congo red-protein complexes. Panel B presents the proteolysis (trypsin, 20(C) of the samples shown in panel A, analyzed by SDS PAGE. 1, 4 – control samples (uncomplexed Lλ); 2, 5 (complexes formed at 20°C; 3, 6 – complexes formed at 45°C; S – molecular weight standards (Sigma, USA).

The binding of Congo red and/or ANS to heat-denatured proteins usually affects the visible spectrum and fluorescence, respectively. The spectral changes involve increased absorbency and a shift of the main absorption band toward red. A similar effect is observed upon dissolving Congo red in alcohols [26]. The effect likely increases with a decrease of the dielectric constant. In the case of Congo red complexation with protein, this results from the dye's penetration to the protein inside. The hydrophobic interior of heated L chain An, according to this test, is impenetrable until about 60°C, while the accessibility of L chain Kok begins at 40°C. The alteration of the Congo red spectrum appears parallel to changes in ANS fluorescence, which is known to depend on hydrophobicity at the site of ANS binding (Fig. 2).

In contrast, almost no or only negligible spectral changes are observed upon complexation of Congo red at room temperature and up to 40°C to myeloma proteins (Fig. 2) even though the complexes formed are definite (Fig. 3A). This can be explained by assuming that the location of Congo red in the domain is not necessarily in the hydrophobic center, but may be more peripheral. Perhaps the most convincing indication that Congo red ligation is conditioned by the instability of polypeptide chain packing, allowing the dye to penetrate, is the effect of its complexation on the increase in immunoglobulin's susceptibility to proteolysis, which also differs for L chain An and Kok (Fig. 3B). This effect may be interpreted as resulting from the replacement of the unstable polypeptide loop by the supramolecular dye ligand at the packing locus, and the resulting exposure to enzymatic attack [27]. At least two structural factors seem to be responsible for the significantly higher instability of the L chain Kok we observed. These involved the improper glycosylation revealed using labeled lectins [28–31], and in particular the Pro13Leu mutation in the conservative β-turn, important for proper packing of the N-terminal polypeptide loop, which is pivotal for the stability of the V domain [32]. This is seen when the amino acid sequence of the polypeptide fragment (21 aa) of these L chains are compared:

```
                    10        20        30
Lλ(An)   SSELTQPPSVSVSPGQXXRITCSGEAVSTHSFAW-
Lλ(Kok)   SELTQDPAVSVALGQTVRITCQGDSIRTIYASWQ
```

A special technique to study Congo red binding to myeloma immunoglobulins was applied in two-dimensional agarose gel electrophoresis. Congo red was introduced to the second run by distributing it below and along the protein fractions developed in the first run. The fast-moving anionic dye overtakes and overruns the migrating proteins, selectively binding those susceptible to dye penetration. This technique allows Congo red-

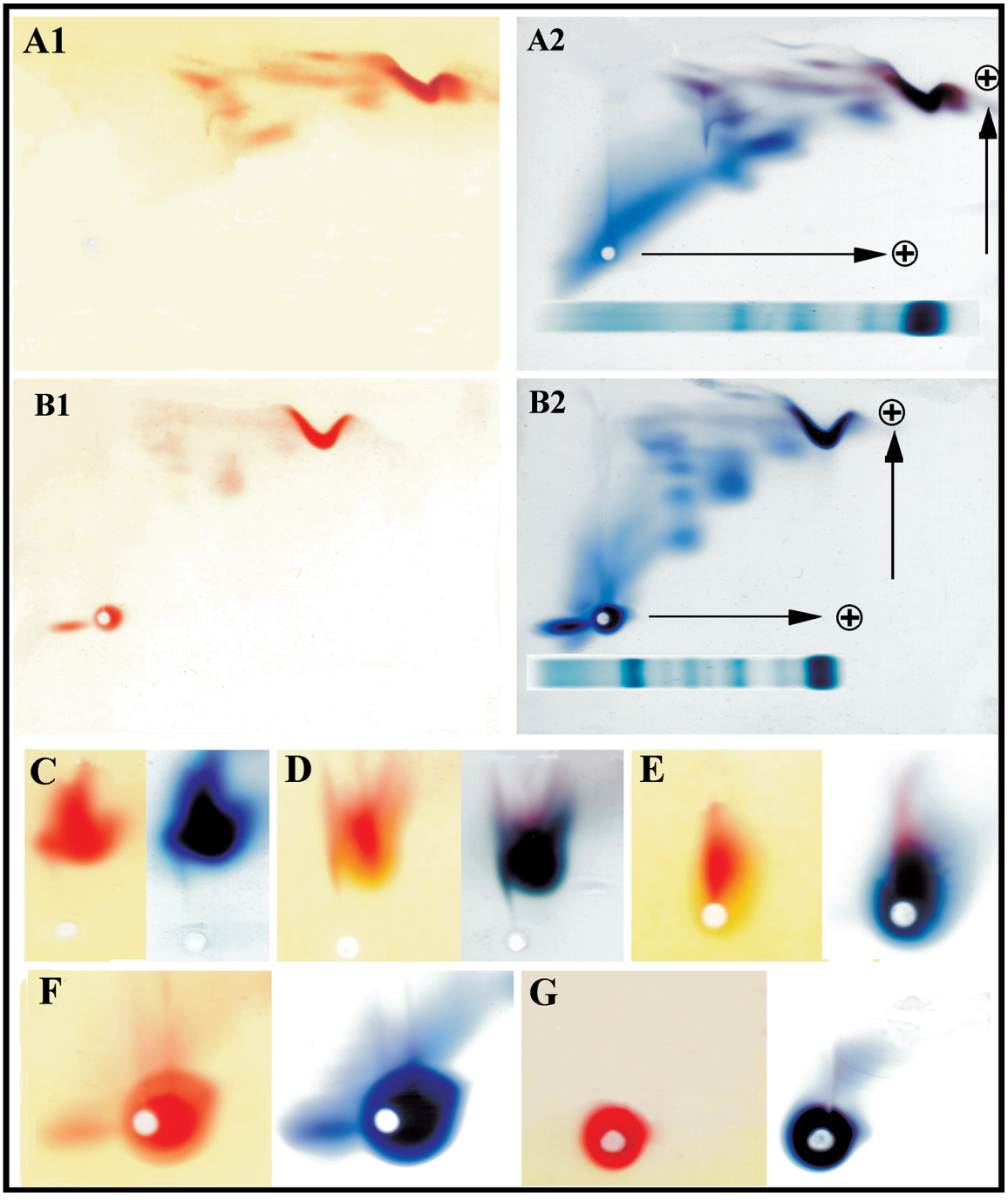


Figure 4. Congo red binding capability of myeloma-derived immunoglobulins disclosed, after separating other serum proteins in two-dimensional agarose electrophoresis. A1, A2 – non-myeloma protein serum electrophoresis with high polyclonal immunoglobulin content, stained respectively with Congo red (A1) and bromophenol blue (A2). B1, B2 – two-dimensional electrophoresis of serum proteins containing myeloma immunoglobulin B1 stained by Congo red, B2 stained by bromophenol blue. Arrows in panel A2 and B2 indicate the direction of electrophoretic runs. One-directional electrophoreses of these sera are inserted at the bottom of panels A2 and B2, respectively. Panels C, D, E, F and G show magnified immunoglobulin spots and their immediate area stained with Congo red (left) and bromophenol blue (right). The presented examples show the different character and behavior of myeloma protein-Congo red complexes in electrophoresis. Protein spots not stained by Congo red but revealed by bromophenol blue, seen in panels D, E, F and G, represent the polyclonal immunoglobulin fraction and nonbonding monoclonal immunoglobulins or their portions. At higher concentrations they are also exposed before bromophenol blue staining as yellow spots resulting from the attachment of picric acid used for protein fixation.

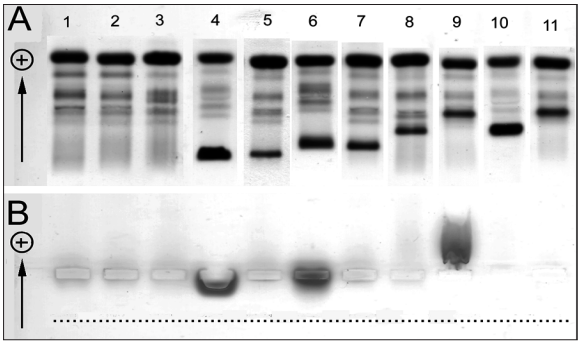


Figure 5. The use of Congo red in the electrophoretic test to differentiate monoclonal proteins of high instability in human myeloma sera. A – standard electrophoresis of sera containing polyclonal IgG (1–3) and monoclonal components IgG and IgA (4–11) stained with bromophenol blue dye. B – The same sera (positioned as in A) analysed in electrophoretic testing for Congo red binding. The sera used for Congo red binding analysis were suitably diluted to bring the myeloma proteins to the same concentration (about 22 g/L). The dotted line shows the position for application of Congo red.

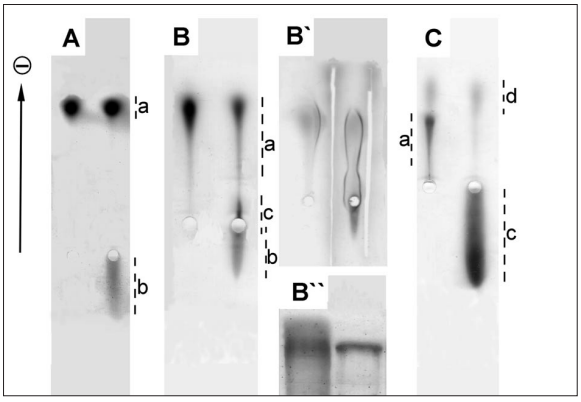


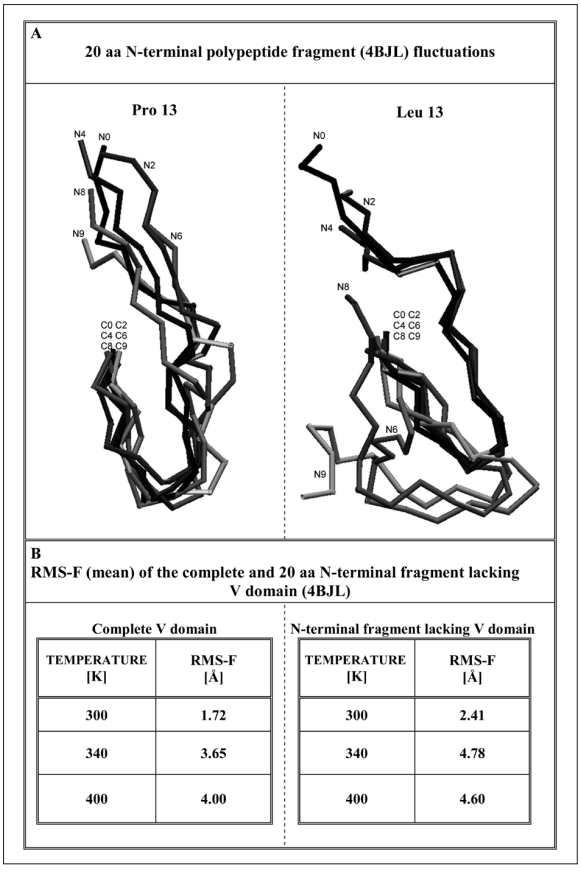
Figure 6. Different reactivities of myeloma IgG with Congo red revealed by reverse electrophoresis in agarose gel with CM-cellulose added to the gel and buffer. Immunoglobulins were isolated by extraction from agarose gels after electrophoretic separation of sera. The right lane in each test represents the electrophoretic behavior of the sample incubated with Congo red. A – non-binding myeloma immunoglobulin; B – non-homogenous (in respect to Congo red binding) myeloma immunoglobulin. (B') non-homogeneity of myeloma IgG from panel B, shown by immunoelectrophoresis with anti-IgG serum; (B'') electrophoretic homogeneity of this protein (pI 7.1) tested by isoelectric focusing (right panel), compared to polyclonal IgG fraction (left panel); C – strongly binding myeloma IgG. a – non-complexed monoclonal immunoglobulin; b – Congo red; c – Congo red-protein complexes; d – polyclonal immunoglobulin fraction. Amido black was used for staining.

binding proteins to be distinguished as stained, extra-charged spots migrating faster than the original molecules [25]. The normal native serum immunoglobulin fraction loses weakly attached dye in the electric field and remains unstained. The adsorption of dye to the immunoglobulin fraction indicates that an abnormal protein component, possibly with an increased propensity to associate, is present in the serum (Fig. 4). Additional staining of the plate with amido black or bromophenol blue dyes, directly or after prior removal of Congo red by reduction with sodium dithionite, reveals a polyclonal immunoglobulin fraction or possibly the presence of myeloma protein not binding Congo red (Figs 4A2, B2). Some serpin and haptoglobin derivatives and complexes formed by these proteins also bind Congo red, but their electrophoretic migration differs significantly and there is no overlap with the immunoglobulin fraction that could interfere with the evaluation of dye binding.

A protein's significantly increased ability to bind Congo red may also be revealed by a screening technique based on unidirectional agarose electrophoresis, which allows for preliminary evaluation of several sera in a single electrophoretic run (Fig. 5). For this purpose the electrophoresis is done in the standard way initially. After a short time to allow the albumin and globulins to separate, Congo red (1.5 mg/mL) is introduced and electrophoresis is continued until the fast-migrating dye overruns the proteins. The plate is then washed for 10 min in 20% methanol and blotted with filter paper (Whatman 3MM), to remove the dye and stained soluble protein electrophoretic fraction from the plate, while the myeloma proteins, strongly binding the dye, remain adsorbed to agarose. Blotting is done with wet filter paper covered by several layers of dry filter paper to enhance the transfer of easily soluble protein components. After blotting, the plate is dried and additionally stained with bromophenol blue dye if necessary.

While decreased stability of the myeloma protein resulting from structural defects seems reasonable and may be expected to involve all molecules of the given myeloma protein, it was surprising to find seemingly typical monoclonal proteins, which, however, showed nonhomogenous reactivity with Congo red despite their homogeneity in standard agarose electrophoresis and even in isoelectric focusing.

Although the two-dimensional electrophoresis described here may sufficiently disclose this phenomenon, an additional electrophoretic variant was developed to verify it. This is standard agarose electrophoresis with soluble CM-cellulose added to the barbiturate buffer and the gel. The charge effect introduced to the electrophoretic bed by CM-cellulose reverses the migration, but with the standard order of protein distribution preserved. As a result, immunoglobulins represent the fraction with the fastest migration toward the cathode, while albumin migration is the slowest. Evaluation of the properties and behavior of the immunoglobulin fraction, which becomes highly mobile, is significantly facilitated. In this electrophoresis, migration of the extra negatively charged immunoglobulin-dye complex is slower than the migration of free protein not engaged in complexation. Despite the highly unfavorable conditions for Congo red-protein complexes in this elec-



Complete V domain	
TEMPERATURE [K]	RMS-F [Å]
300	1.72
340	3.65
400	4.00

N-terminal fragment lacking V domain	
TEMPERATURE [K]	RMS-F [Å]
300	2.41
340	4.78
400	4.60

Figure 7. Effect of defective N-terminal polypeptide chain structure and its deficient packing on V domain stability. A – Overmobility of 20 aa N-terminal polypeptide chain fragment resulting from Pro13Leu exchange, revealed by simulation of molecular dynamics in model studies (based on 4BJL PDB notation). Consecutive steps in time show the post-dynamic structures after 0, 2, 4, 6, 8 and 9 ns, respectively, represented by stick models additionally differentiated by alteration of blackening (decreasing in time). B – Essential decrease of V domain stability in Lλ (4BJL) deprived of 20 aa N-terminal chain fragment versus the complete V domain, revealed by simulation of molecular dynamics as increased fluctuation. The effect is represented as the root mean square fluctuation values (RMS-F) averaged for V domains at increasing temperature. RMS-F V – values for the complete V domain; RMS-F V' – values for N-terminal peptide-deprived V domain.

trophoretic system, their stability is usually enough to affect migration and they can easily be registered (Fig. 6).

Conclusions

The ribbon-shaped structures of strongly associated Congo red molecules easily adhere to cellulose and other chain-like periodic polymers, including β-conformation polypeptides [16,17,19]. The clefts formed transiently in β-structural protein fragments with decreased stability, which become accessible to penetration by foreign compounds and offer direct contact with polypeptide backbone interfaces, are suitable receptor sites for supramolecular Congo red ligands. While heating is necessary to make normal native immunoglobulin molecules accessible for Congo red complexation, many molecules produced by cancer cells appear to have abnormal structure, making them significantly unstable and hence accessible for dye attachment even at room temperature.

In general, two defective steps of immunoglobulin biosynthesis may be responsible for the instability of these molecules: (1) improper recombination or hypermutation [33–37] and (2) transcriptional or posttranscriptional alterations [38,39]. The first may be expected to produce monoclonal proteins that are homogenous in respect to Congo red binding, while structural deviations originating from posttranscriptional steps may not be homogenous, as an effect of only partial involvement of proper RNA processing, polypeptide chain folding or glycosylation.

Congo red was chosen as a probe to register the decreased stability of myeloma proteins. The mechanism of Congo red complexation with proteins is still not understood unequivocally. It is likely conditioned by the complicated liquid crystalline character of this dye, its polymorphic form in water solutions and different possible interactions [40]. However, the most energetically optimal supramolecular dye arrangement seems to result from face-to-face stacking of molecules [15–17], which produces ribbon-shaped micellar entities easily adhering to linear polymers. This most probably determines their specific affinity to β-structure.

Congo red interaction may also involve nonspecific surface attachment to protein. However, the complexation products that persist in standard agarose electrophoresis or gel filtration are likely formed by adhesion of supramolecular dye to polypeptide backbone interfaces, after penetration into crevices formed in unstable protein β-structural regions [22]. The conformation-dependent affinity also explains the interaction of amyloid deposits with Congo red, commonly considered to be specific for these protein forms, in spite of their different origins and amino acid sequences [41–45]. Studies addressing the mechanism of Congo red interaction with amyloid proteins have also supplied some evidence that the polypeptide conformation and the particular supramolecular dye organization may determine binding specificity [46,47], rather than the specific amino acid combination which is suggested for single dye molecule surface attachment [48–50].

Congo red's ability to bind myeloma proteins was not observed to diminish upon dilution, although protein association at high density may favor dye complexation by arresting transiently unpacked conformations [51]. This would confirm intra- rather than intermolecular dye binding.

Disclosure of Congo red-immunoglobulin complexes persistent in electrophoresis and/or gel filtration may represent a simple general test for protein instability.

The ability to register dye anchorage at the periphery of the protein of the still impenetrable hydrophobic core

significantly enlarges the range of diagnostic analysis of abnormalities in myeloma immunoglobulins [52–54]. Most of the studied myeloma proteins do not affect ANS fluorescence at room temperature, while Congo red complexation is already clearly registered. Congo red may thus close the gap in the registration of packing deviations of the polypeptide chain, that is, the gap between the normal native immunoglobulin fold and that which corresponds to the molten globule [20].

Theoretical model studies help to understand how local packing instability makes the V domain accessible for Congo red binding. These studies have involved the instability caused by the Pro13Leu mutation (Fig. 7A), and show the difference in mobility during 9 ns simulation of the 21 aa N-terminal fragment with Pro or Leu at the conservative β-turn in the V domain (4BJL PDB file). The inserted table (Fig. 7B) represents the averaged RMS-fluctuation (RMS-F) data concerning domain mobility calculated for the complete L chain, and the same chain deprived of the 21 aa N-terminal fragment, indicating the importance of this fragment for domain stability.

The electrophoretic technique used in this work discloses, besides myeloma immunoglobulins, several other proteins in serum which appear to bind Congo red: mostly serpin derivatives, serpin complexes, β-lipoprotein, and some as-yet-unidentified protein serum components. These may participate in aggregation phenomena, and seem to explain the observed nonhomogeneity of Alzheimer plaque and amyloid deposits [55–60].

The expanding field of protein aggregation-derived pathology requires new specific clinical tests for analysis. The method proposed here offers a step forward. The identification of dye-binding myeloma protein in serum may have diagnostic value in revealing potential pathogenic activity.

CONCLUSIONS

Many myeloma proteins bind supramolecular dye Congo red. The complexes formed by Congo red with these proteins differ in stability. Those of high stability indicate abnormal protein structure, expected to produce clinical symptoms. This work proposes an easy electrophoretic technique allowing the stability of complexes to be differentiated.

REFERENCES:

1. Cogné M, Silvain C, Khamlichi AA, Preud'homme JL: Structurally abnormal immunoglobulins in human immunoproliferative disorders. Blood, 1992; 79: 2181
2. Buxbaum JN: Abnormal immunoglobulin synthesis in monoclonal immunoglobulin light chain and light and heavy chain deposition disease. Amyloid: J Protein Folding Disord, 2001; 8: 84
3. Landsbury PT: Evolution of amyloid: What normal protein folding may tell us about fibrillogenesis and disease. Proc Natl Acad Sci USA, 1999; 96: 3342
4. Attaelmannan M, Levinson SS: Understanding and identifying monoclonal gammopathies. Clin Chem, 2000; 46: 1230
5. Wall J, Schell M, Murphy C et al: Thermodynamic instability of human λ6 light chains: Correlation with fibrillogenecity. Biochemistry, 1999; 38: 14101
6. Wetzel R: Domain stability in immunoglobulin light chain deposition disorder. Adv Protein Chem, 1997; 50: 183
7. Dobson CM: The structural basis of protein folding and its links with human disease. Philos Trans R Soc Lond B Biol Sci, 2001; 356: 133
8. Kim YS, Wall JS, Meyer J et al: Thermodynamic modulation of light chain amyloid fibril formation. J Biol Chem, 2000; 275: 1570
9. Khurana R, Gillespie JR, Talapatra A et al: Partially folded intermediates as critical precursors of light chain amyloid fibrils and amorphous aggregates. Biochemistry, 2001; 40: 3525
10. Privalov PL, Gill SJ: Stability of protein structure and hydrophobic interaction. Adv Protein Chem, 1988; 39: 191
11. Goto Y, Fink AL: Conformational states of β-lactamase: molten-globule states at acidic and alkaline pH with high salt. Biochemistry, 1989; 28: 945
12. Lattman EE, Fiebig KM, Dill KA: Modeling compact denatured states of proteins. Biochemistry, 1994; 33: 6158
13. Ptitsyn OB, Bychkova VE, Uversky VN: Kinetic and equilibrium folding intermediates. Philos Trans R Soc Lond B Bio Sci, 1995; 348: 35
14. Roterman I, No KT, Piekarska B et al: Bis azo dyes - Studies on the mechanism of complex formation with IgG modulated by heating and antigen binding. J Physiol Pharmacol, 1993; 3: 215
15. Skowronek M, Stopa B, Konieczny L et al: Self-assembly of Congo red - A theoretical and experimental approach to identify its supramolecular organization in water and salt solution. Biopolymers, 1998; 46: 267
16. Skowronek M, Roterman I, Konieczny L et al: Why do Congo red, Evans blue and Trypan blue differ in their complexation properties? J Comput Chem, 2000; 21: 656
17. Roterman I, Rybarska J, Konieczny L et al: Congo red bound to α-1-proteinase inhibitor as a model of supramolecular ligand and protein complex. Comput Chem, 1998; 22: 61
18. Stopa B, Górny M, Konieczny L et al: Supramolecular ligands: Monomer structure and protein ligation capability. Biochimie, 1998; 80: 963
19. Roterman I, Król M, Nowak M et al: Why Congo red binding is specific for amyloid proteins - model studies and a computer analysis approach. Med Sci Monit, 2001; 7: 771
20. Piekarska B, Skowronek M, Rybarska J et al: Congo red-stabilized intermediates in the λ light chain transition from native to molten state. Biochimie, 1996; 78: 183
21. Stopa B, Konieczny L, Piekarska B et al: Effect of self association of bis-ANS and bis-azo dyes on protein binding. Biochimie, 1997; 79: 23
22. Piekarska B, Konieczny L, Rybarska J et al: Heat-induced formation of a specific binding site for self-assembled Congo red in the V domain of immunoglobulin L chain l. Biopolymers, 2001; 59: 446
23. Roterman I, Konieczny L, Stopa B et al: Heat-induced structural changes in the Fab fragment of IgG recognized by molecular dynamics simulation - Implications for signal transduction in antibodies. Folia Biol (Krakow), 1994; 42: 115
24. Rybarska J, Konieczny L, Roterman I, Piekarska B: The effect of azo dyes on the formation of immune complexes. Arch Immunol Ther Exp, 1991; 39: 317
25. Rybarska J, Konieczny L, Piekarska B et al: The detection of specific acute phase serum protein complexes and immune complexes by Congo red binding. J Physiol Pharmacol, 1995; 46: 221
26. Piekarska B, Roterman I, Rybarska J et al: The melting of native domain structure in effector activation of IgG studied by using Congo red as a specific probe. J Physiol Pharmacol, 1994; 45: 147
27. Rybarska J, Piekarska B, Konieczny L, Roterman I: The formation of soluble heat IgG aggregates for immunological studies. Arch Immunol Ther Exp, 1988; 36: 609
28. Leibiger H, Wüstner D, Stigler RD, Marx U: Variable domain-linked oligosaccharides of a human monoclonal IgG: structure and influence on antigen binding. Biochem J, 1999; 338: 529
29. Foss GS, Nilsen R, Cornwell III GG et al: A glycosylated Bence Jones protein and its autologous amyloid light chain containing potentially amyloidogenic residues. Scand J Immunol, 1998; 47: 348

30. Krotkiewski H: Carbohydrate moiety of immunoglobulins in health and pathology. Acta Biochim Pol, 1999; 46: 341
31. Stevens FJ: Four structural risk factors identify most fibril-forming kappa light chains. Amyloid: Int J Exp Clin Invest, 2000; 7: 200
32. Ohage E, Steipe B: Intrabody construction and expression I. The critical role of VL domain stability. J Mol Biol, 1999; 291: 1119
33. Goossens T, Klein U, Küppers R: Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. Proc Natl Acad Sci USA, 1998; 95: 2463
34. Willis TG, Dyer MJS: The role of immunoglobulin translocations in the pathogenesis of B-cell malignancies. Blood, 2000; 96: 808
35. Bross L, Fukita Y, McBlane F et al: DNA double-strand breaks in immunoglobulin genes undergoing somatic hypermutation. Immunity, 2000; 13: 589
36. Williams GT, Jolly CJ, Köhler J, Neuberger MS: The contribution of somatic hypermutation to the diversity of serum immunoglobulin: dramatic increase with age. Immunity, 2000; 13: 409
37. Milstein C, Neuberger MS, Staden R: Both DNA strands of antibody genes are hypermutation targets. Proc Natl Acad Sci USA, 1998; 95: 8791
38. Helenius A: Quality control in the secretory assembly line. Philos Trans R Soc Lond B Biol Sci, 2001; 356: 147
39. Bross P, Corydon TJ, Andresen BS et al: Protein misfolding and degradation in genetic diseases. Hum Mutat, 1999; 14: 186
40. Khurana R, Uversky VN, Nielsen L, Fink AL: Is Congo red an amyloid-specific dye? J Biol Chem, 2001; 276: 22715
41. Janek K, Behlke J, Zipper J et al: Water-soluble β-sheet models which self-assemble into fibrillar structures. Biochemistry, 1999; 38: 8246
42. Chiti F, Webster P, Taddei N et al: Designing conditions for *in vitro* formation of amyloid protofilaments and fibrils. Proc Natl Acad Sci USA, 1999; 96: 3590
43. West MW, Wang W, Patterson J et al: De novo amyloid proteins from designed combinatorial libraries. Proc Natl Acad Sci USA, 1990; 96: 11211
44. Agueda R, Ruben V, Batia K et al: pH-dependent fibrillogenesis of a V [kappa]III Bence Jones protein. Br J Haematol, 1999; 107: 835
45. Orpiszewski J, Benson MD: Induction of β-sheet structures in amyloidogenic peptides by neutralization of aspartate: A model for amyloid nucleation. J Mol Biol, 1999; 289: 413
46. Pollack SJ, Sadler IJ, Hawtin SR et al: Sulfonated dyes attenuate the toxic effects of β-amyloid in a structure-specific fashion. Neurosci Lett, 1995; 197: 211
47. Rybarska J, Piekarska B, Stopa B et al: Evidence that supramolecular Congo red is the sole ligation form of this dye for L chain λ derived amyloid proteins. Folia Histochem Cytobiol, 2001; 39: 307
48. Li L, Darden TA, Bartolotti L et al: An atomic model for the pleated β-sheet structure of Aβ amyloid protofilaments. Biophys J, 1999; 76: 2871
49. Klunk WE, Debnath ML, Pettegrew JW: Development of small molecule probes for the β-amyloid protein of Alzheimer's Disease. Neurobiol Aging, 1994; 13: 691
50. Carter DB, Chou K: A model for structure-dependent binding of Congo red to Alzheimer β-amyloid fibrils. Neurobiol Aging, 1998; 19: 37
51. Muramatsu N, Minton AP: Hidden self-association of proteins. J Mol Recognit, 1989; 1: 166
52. Chothia C: Principles that determine the structure of proteins. Annu Rev Biochem, 1984; 53: 537
53. Janin J, Miller S, Chothia C: Surface, subunit interfaces and interior of oligomeric proteins. J Mol Biol, 1988; 204: 155
54. Chothia C, Gelfand I, Kister: Structural determinants in the sequences of immunoglobulin variable domain. J Mol Biol, 1998; 278: 457
55. Serpell LC, Sunde M, Blake CCF: The molecular basis of amyloidosis. Cell Mol Life Sci, 1997; 53: 871
56. Pepys MB: Pathogenesis, diagnosis and treatment of systemic amyloidosis. Philos Trans R Soc Lond B Biol Sci, 2001; 356: 203
57. McLaurin J, Yang DS, Yip CM, Fraser PE: Modulating factors in amyloid-β fibril formation. J Struct Biol, 2000; 130: 259
58. Fraser PE, Darabie AA, McLaurin: Amyloid-β interactions with chondroitin sulfate-derived monosaccharides and disaccharides. J Biol Chem, 2001; 276: 6412
59. Eriksson S, Janciauskiene S, Lannfelt L: α-1-antichymotrypsin regulates Alzheimer β-amyloid peptide fibril formation. Proc Natl Acad Sci, 1995; 92: 2313
60. Martin GM: Molecular mechanism of late life dementias. Exp Gerontol, 2000; 35: 439