Is Congo red an amyloid specific dye?

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Abstract

Congo red (CR) binding, monitored by characteristic yellow-green birefringence under crossed polarization has been used as a diagnostic test for the presence of amyloid in tissue sections for several decades. This assay is also widely used for the characterization of in vitro amyloid fibrils. In order to probe the structural specificity of Congo red binding to amyloid fibrils we have used an induced circular dichroism (CD) assay. Amyloid fibrils from insulin and the variable domain of Ig light chain demonstrate induced CD spectra upon binding to Congo red. Surprisingly, the native conformations of insulin and Ig light chain also induced Congo red circular dichroism, but with different spectral shapes than those from fibrils. In fact, a wide variety of native proteins exhibited induced CR circular dichroism indicating that CR bound to representative proteins from different classes of secondary structure such as α (citrate synthase), α+β (lysozyme), β (Concavalin A) and parallel β-helical proteins (pectate lyase). Partially-folded intermediates of apomyoglobin induced different Congo red CD bands than the corresponding native conformation, however, no induced CD bands were observed with unfolded protein. Congo red was also found to induce oligomerization of native proteins, as demonstrated by covalent crosslinking and small angle X-ray scattering. Our data suggest that Congo red is sandwiched between two protein molecules causing protein oligomerization. The fact that Congo red binds to native, partially folded conformations and amyloid fibrils of several proteins shows that it must be used with caution as a diagnostic test for the presence of amyloid fibrils in vitro.
Abbreviations:

CR – Congo red; CD - Circular Dichroism; ORD - optical rotary dispersion; DTSSP - [3,3’-Dithiobis (sulfosuccinimidylpropionate)]; DSP [Dithiobis(succinimidylpropionate)]; SMA initials of the patient with light chain amyloidosis whose sequence information was used to generate synthetic recombinant proteins by Stevens et al (1995); ATR- Attenuated total reflectance; FTIR - Fourier transform infrared spectroscopy; SDS – sodium dodecyl sulfate; PAGE – polyacrylamide gel electrophoresis SAXS - Small angle X-ray scattering; ANS - 1, 8-anilino-naphthalene sulfonate
Introduction

In the 1920's Benhold and Divry established that Congo red bound to amyloid in tissue sections and demonstrated its characteristic yellow-green birefringence under crossed polarizers. Since then this birefringence has been used as a diagnostic for amyloid fibrils. The birefringence assay is not a simple one, for example, the tissue sections need to be of a required thickness to show birefringence, reviewed by Elghetany and Saleem and Westermark. Congo red binding is not specific for amyloid in the tissue sections, but the assays are performed under extreme conditions with 50 – 80 % ethanol, high salt and alkaline pH conditions to yield binding to amyloid. Despite these extreme conditions, binding to collagen fibers and cytoskeletal proteins results in false positive results. Due to the difficulties with the birefringence assay for in vitro amyloid fibrils, Klunk and coworkers have developed simpler filtration-based assays followed by measuring the concentration of free Congo Red to quantify dye binding. The filtration assays would not detect CR bound to soluble monomers or oligomers as they are not large enough to be trapped by 0.2 μm filters. Large particles, such as amyloid fibrils, are retained on the filters accounting for the loss of free dye molecules, whereas any native protein molecules bound to CR would pass through the filter pores. Thus the filtration assay is not affected by possible binding of CR to native soluble conformations of protein.

Benditt and coworkers analyzed spectral probes such as absorbance red shift, optical rotatory dispersion (ORD) and circular dichroism (CD) to describe the Cotton effect responsible for birefringence. They used human albumin, poly-L-lysine with different conformations and amyloid fibrils as substrates for binding of Congo red. The random coil conformation of poly-L-
lysine did not show spectral changes, but both helical and \( \beta \) conformations induced Congo red CD bands as well as ORD spectra similar to those observed with the amyloid fibril samples.

Edwards and Woody \cite{10,11} demonstrated that induced circular dichroism can be used as a probe for Cibacron blue and Congo red bound to dehydrogenases such as liver alcohol dehydrogenase, yeast alcohol dehydrogenase, lactic dehydrogenase, and kinases including phosphoglycerate kinase and porcine adenylate kinase. Edwards and Woody \cite{11} believed that Congo red bound to the coenzyme binding sites of the enzymes based on the similarities between dye and coenzyme structures. Congo red has also been shown to bind other native proteins including cellular prion protein \cite{12}, elastin \cite{13}, RNA polymerase \cite{14} and human prostatic phosphatase \cite{15}.

The structure of Congo red,

\[
\begin{align*}
\text{NH}_2 & \quad N=N & \quad N = N & \quad N = N & \quad \text{NH}_2 \\
& \quad \text{SO}_3\text{Na} & \quad \text{SO}_3\text{Na} \\
& \quad \text{SO}_3\text{Na} & \quad \text{SO}_3\text{Na}
\end{align*}
\]

suggests that binding to protein could occur through a combination of both hydrophobic and electrostatic interactions. An additional complication is that CR has been reported to form linear ribbon-like micelles. \cite{16,17} In an attempt to understand the binding specificity of Congo red we have used induced circular dichroism as an assay for binding of CR to native proteins, partially folded protein conformations and amyloid fibrils, using native proteins from a variety of different secondary structure classes. The results suggest a mechanism of binding of Congo red
to native proteins involving the intercalation of Congo red between protein molecules leading to oligomerization of the protein.

**Materials and Methods**

*Fibril formation*

Fibril were grown in vitro with a 0.5 mg/ml SMA (a recombinant amyloidogenic variable domain of Ig light chain made by Stevens and coworkers\textsuperscript{18}) and 1 mg/ml bovine insulin solution at pH 2 in 20 to 50 mM HCl and 100 mM NaCl, that were agitated using a magnetic stirrer in a 37 °C incubator for a day.

*CD measurements*

CD spectra were collected between 650 nm and 300 nm, with 1 nm step size, 10 s averaging time, in an Aviv 62 DS spectropolarimeter. Induced CD spectra were obtained using a split quartz cell with each compartment having a path length of 5 mm and total path length of 10 mm. The protein and Congo red solutions of twice the final concentration were added in each compartment to obtain a control spectrum. Induced circular dichroism was only observed upon mixing the Congo red and the protein solutions before collecting the spectrum again. The induced CD assay was performed at pH 7.5 using a 1 cm path length rectangular cell with final Congo red concentration of 30 to 40 µM with protein concentration of 0.1 to 0.2 mg/ml. At pH 2.0 the induced CD assay was performed using a 10 cm path length circular CD cell with final Congo red concentrations of 3 - 4 µM as the solubility of Congo red was much lower at pH 2. Induced CD measurements for fibril samples were obtained with a 10 cm pathlength circular cell using very dilute samples (80 to 400 nM), as at higher concentrations the fibril samples precipitated as red particles.
Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

Hydrated (H₂O) thin film spectra were collected using a Nicolet 800 FTIR spectrometer equipped with a liquid nitrogen cooled MCT detector and purged with dry air. All samples were scanned in an out-of-compartment horizontal ATR accessory (SPECAC) with a high throughput 73 x 10 x 6 mm, 45° trapezoidal germanium crystal (the internal reflectance element). To collect spectra protein samples with and without Congo red were applied (40 µl of 1mg/mL protein solution and 500 µM Congo red) and dried while being spread constantly with a spatula. Data processing was done with GRAMS32 (Galactic Industries) and SAFAIR software as previously described 19. Water vapor components were subtracted until the region between 1800 to 1700 wavenumbers was featureless.

Analysis of binding constants and number of binding sites

Equation 1 was used to fit the data obtained at 40 µM Congo red and varying the protein concentration from 0 to 20 µM and measuring the CD spectra of protein bound dye molecules.

\[
C = n C_m \frac{[L]}{[L] + K_d}
\] (1)

where C is the change in circular dichroism signal at a particular wavelength measured upon addition of protein ([L]) in varying concentration, C_m is the maximal CD change obtained by varying the protein concentration at a constant Congo red concentration, n is the number of binding sites for Congo red on the protein and K_d is the dissociation constant for binding of Congo red to protein.
Crosslinking and SDS-PAGE analysis

10 μM DTSSP [3,3′-Dithiobis(sulfosuccinimidylpropionate)], a bifunctional, cleavable crosslinking reagent, was added to the protein solution in the absence and presence of Congo red and incubated for 1 hour. DTSSP (Pierce) is a sulfonated derivative of DSP [Dithiobis(succinimidylpropionate)] that is water soluble, unlike DSP, and, like DSP, can be cleaved under reducing conditions (the presence of DTT or β-mercaptoethanol). The crosslinked protein was then precipitated using 10 % acetic acid, and spun in a centrifuge. The pellet was resuspended in SDS loading buffer and separated on either a non-reducing or a reducing 8-25 % SDS-PAGE gel.

Small-angle X-ray scattering experiments

Small angle X-ray scattering (SAXS) measurements were done using Beam Line 4-2 at Stanford Synchrotron Radiation Laboratory (Wakatsuki et al., 1992). X-ray energy was selected at 8980 eV (Cu edge) by a pair of Mo/B_{4}C multilayer monochromator crystals (Tsuruta et al., 1998). Scattering patterns were recorded by a linear position-sensitive proportional counter, which was filled with an 80% Xe / 20% CO_{2} gas mixture. Scattering patterns were normalized by incident X-ray fluctuations, which were measured with a short length ion chamber before the sample. The sample-to-detector distance was calibrated to be 230 cm, using a cholesterol myristate sample. The measurements were performed in a 1.3 mm path length observation static cell with 25 μm mica windows. To avoid radiation damage of the protein samples during the SAXS measurements, the scavenger N-tert-butyl-α-(4-pyridyl)-nitrone-N’-oxide was added to a final concentration of 10 mM. Background measurements were performed before and after each
protein measurement and then averaged for background subtraction. All SAXS measurements were performed at 23±1 °C.

The radius of gyration ($R_g$) was calculated according to the Guinier approximation (Glatter & Kratky, 1982):

$$\ln I(Q) = \ln I(0) - R_g^2 Q^2/3$$

(2)

where $Q$ is the scattering vector given by $Q = (4\pi \sin \theta)/\lambda$ ($2\theta$ is the scattering angle, and $\lambda$ is the wavelength of X-ray). $I(0)$, the forward scattering amplitude, is proportional to $n \cdot \rho c^2 \cdot V^2$ where $n$ is the number of scatters (protein molecules) in solution; $\rho c$ is the electron density difference between the scatter and the solvent; and $V$ is the volume of the scatter. This means that the value of forward-scattered intensity, $I(0)$, is proportional to the square of the molecular weight of the molecule (Glatter & Kratky, 1982). Thus, $I(0)$ for a pure $N$-mer sample will therefore be $N$-fold that for a sample with the same number of monomers since each $N$-mer will scatter $N^2$ times as strongly as monomer, but in this case the number of scattering particles ($N$-mers) will be $N$ times less than that in the pure monomer sample.

**Estimation of the shape of particles using SAXS data**

The relationship between $R_S$ (Stokes radius) and $R_g$ (radius of gyration) is quite sensitive to the shape and compactness of protein, as $R_s/R_g = P^{1/3} \left[5/(P^2 + 2)\right]^{1/2}$, where $P = a/b$, and $a$ and $b$ are the semiaxis of the revolution of the ellipsoid and the equatorial radius of the ellipsoid, respectively. In order to estimate the $R_S$ value for the oligomeric form of $\beta$-lactoglobulin the following approach was used. SAXS data show that in the presence of 1 mM Congo red $\beta$-lactoglobulin forms 28-mers, which corresponds to the particles with the molecular mass of 515,200 Da ($= 28$
x 18,400 Da). The value of $R_S$ of a globular protein with the molecular mass of 515,200 Da is 71.4 Å. This was calculated from an empirical equation $\log(R_S^N) = 0.369 \cdot \log(M) - 0.254$ 23 based on the intrinsic viscosity data 24. Here $R_S^N$ is the Stokes radius of native (N) protein. It is known that for an ideal spherical particle $a=b$, $P=a/b=1.00$ and $R_S/R_g=1.29$, whereas for globular proteins the average value of this ratio is about 1.25 25.

Results and Discussion

Amyloid Fibrils and native conformations show different induced Congo red CD bands

Amyloid fibrils made in vitro from purified proteins such as bovine insulin and Ig light chain (SMA), were tested for binding to Congo red using the apple-green birefringence under crossed-polarization (data not shown) and induced CD signal (Figure 1A). Low concentrations of fibril solutions were used for induced CD measurements, as higher concentrations yielded red precipitates. Both fibril samples showed major induced CD bands with positive maxima in the vicinity of 570 nm and negative maxima in the vicinity of 500-525 nm. The spectrum for the induced CD of insulin fibrils was red-shifted relative to that for the light chain fibrils. The shape of the induced CD bands for Congo red bound to fibrils is different from that described by Benditt et al 8 for tissue extracted amyloid. The differences are most likely due to “contaminants” such as glycosaminoglycans, serum amyloid protein etc. present in the ex-vivo extracts. Native insulin and native SMA show induced Congo red CD bands that differ in shape and intensities from the CD bands induced upon binding to the fibrils (Fig 1B). Instead of a positive and a negative peak observed for the corresponding amyloid fibrils the native proteins show a broad positive band between 500 and 600 nm, that possibly has several components. The peculiar CD band shape with a maxima and a minima is related to the special nature of birefringence 26.
observed for amyloid fibrils upon binding to Congo red. The positive peaks induced by the native proteins were 10-fold smaller in intensity compared to their corresponding amyloid fibrils. A probable explanation is that amyloid fibrils have more binding sites for Congo red than the native protein. The intensity of the induced Congo red CD bands upon binding to native SMA was much smaller than that for native insulin. It is possible that the hexameric insulin (under native conditions) has more Congo red binding sites, leading to a larger induced CD signal, compared to native SMA.

*Congo red binds to native proteins from different secondary structure classes*

Native proteins such as lysozyme (α+β), concavalin A (β) and citrate synthase (all α), (Figure 2) showed induced Congo red CD signals, thus revealing binding of Congo red. A number of other native proteins from different structural classes, interleukin-2 (all α), malate dehydrogenase (α/β), β-lactoglobulin, and apomyoglobin (α) also showed induced Congo red CD signals (data not shown). Other than the observation that the major induced CD bands were in the vicinity of 525 nm, and quite broad, no consistent patterns were apparent. Circular dichroism bands in the visible region were not observed for protein alone, or Congo red alone. This was demonstrated by using split cells where one side of the cell contains the protein solution and the other side of the cell contains Congo red solution, no CD bands were observed until the protein and Congo red solutions were mixed together. Interestingly, no Congo red CD bands were induced in the presence of unfolded protein (e.g. acid unfolded apomyoglobin; Figure 5), amorphous aggregates of P22 tail spike protein, or Ig light chain variable domain, or inclusion bodies. These results are supported by a report that there are no Congo red CD or ORD signals with the random coil conformation of poly-L-lysine⁸. Whereas the induced CD bands indicate a specific orientation of Congo red, which is assumed to be due to binding of the dye to the protein, the
absence of an induced CD band does not necessarily mean the absence of dye binding, but rather the lack of specific orientation responsible for the induced CD bands.

Given that unfolded protein did not induce Congo red CD bands, we conclude that significant secondary structure, and probably a collapsed conformation is required to induce a specific orientation of Congo red molecules responsible for CD bands. Indeed Turnell and Finch observed a Congo red molecule intercalated between the antiparallel β-strands of two insulin molecules, using X–ray diffraction of a crystalline complex. However, our results suggest that Congo red binding is not limited to β-sheet proteins; rather, all classes of proteins, α-helical, β and α+β, also bind Congo red resulting in the observed induced CD bands. Congo red binding to dehydrogenases of the α/β class has been reported previously, and is thought to reflect specific interactions with coenzyme binding sites, due to structural similarities between CR and the coenzyme. β-Helical proteins also induced Congo red CD bands (Figure 3). Interestingly, the right-handed β-helical proteins including pectate lyase and p22 tailspike protein induced different Congo red CD bands, with positive ellipticity, compared to the left-handed β-helical protein LpxA, which induced two negative Congo red CD bands. This suggests that the positive or negative CD bands may reflect the underlying chirality of the CR binding site.

In view of the fact that the induced CD spectra demonstrate that proteins from all classes of secondary structures bind Congo red, Congo red binding is clearly not restricted to the crossed-β structures present in amyloid fibrils. Benditt and coworkers have also shown binding of Congo red to both α and β conformations of poly-L-lysine. It is possible that the shape of the induced Congo red CD bands has specific clues as to which secondary structures in the proteins Congo red dye is bound, but more work is needed to understand these distinctions. The lack of
correlation between the shape of the induced CD band and the protein secondary structure suggests that binding sites for CR in individual proteins are more related to their specific environment rather than to a particular type of secondary structure.

*No secondary structure changes observed in proteins upon binding of Congo red*

Since it has been suggested that Congo red bound specifically to crossed-β structures present in amyloid fibrils, or to β-sheets in native proteins, and our results indicated that all-α proteins also bind Congo red, we sought to confirm that Congo red binding does not induce changes in secondary structure, e.g. from α to β. To test this we collected infrared spectra of interleukin-2 (IL-2) (a four-helix bundle protein) in the absence and presence of Congo red (Figure 4A). The spectrum of Congo red alone was featureless in the amide I and II regions where proteins show specific conformational-sensitive bands, and no secondary structure changes were observed in the IL-2 FTIR spectrum upon binding to Congo red (Figure 4A). Lysozyme, an α+β protein, forms a red precipitate upon binding to Congo red. To test if this precipitate involves formation of new β-structure with low wavenumber amide I peaks, as observed for many protein aggregates (Khurana, Oberg, Sheshadri, Li and Fink manuscript submitted), we examined it with ATR FTIR. The FTIR spectra of soluble free lysozyme and precipitated Congo red-bound lysozyme are compared in Figure 4B. No significant increase in β-structure was observed in the precipitated Congo red-bound lysozyme compared to native lysozyme. The minor differences observed between 1610 and 1580 cm⁻¹ are probably due to interaction of Congo red with specific side chains, as these bands have significant contributions from side chains and are not indicative of protein secondary structure changes. Thus it is clear that binding of CR does not result in induction of β-structure.
Native and partially folded conformations induce different Congo red CD bands

1, 8, anilino-naphthalene sulfonate (ANS) \(^{33}\) and its dimer bis-ANS \(^{34}\) are commonly used as probes of hydrophobic regions in native and partially folded proteins. Due to the similarity of the structures of Congo red and bis-ANS we decided to also test differential binding of Congo red to native and partially folded conformations. Apomyoglobin exists in its native conformation at pH 7, the acid unfolded state at pH 2 in the absence of salts and as a partially folded intermediate at pH 4 \(^{35,36}\) and at pH 2 in the presence of salt \(^{37}\). No induced Congo red CD bands were observed for the acid unfolded form, but the native (pH 7) and partially folded conformations at pH 4 and pH 2 with 500 mM KCl showed different induced spectra (Figure 5). Binding of the dye to the native conformation of apomyoglobin is not surprising, since the protein is known to bind a variety of hydrophobic molecules in the vacant heme-binding site. Increased binding of Congo red has been observed for the molten globule intermediate compared to the native conformation for human prostatic phosphatase (7 to 8 molecules of Congo red bind to the intermediate conformation as opposed to 1.6 dye molecules to the native protein). \(^{15}\) Consequently it appears that CR binding sites are present in partially-folded intermediates. This is not surprising since such intermediates are known to have exposed hydrophobic patches and bind hydrophobic molecules. Thus it is likely that different binding sites for CR may exist in native and partially-folded intermediate states.

Probing the mechanism of Congo red binding

The number of molecules of Congo red bound per molecule of native β-lactoglobulin was estimated from an analysis of the data obtained by varying the concentration of protein from 0 to 20 µM while keeping the concentration of Congo red constant at 40 µM (Figure 6A). The
ellipticity at 530 nm and 450 nm were plotted against \( \beta \)-lactoglobulin concentration (Figure 6B) and the data were fitted to equation 1. The analysis showed that \( 1.52 \pm 0.05 \) molecules of Congo red bound per molecule of \( \beta \)-lactoglobulin, similar to the value obtained by Kuciel and Mazurkiewicz \(^{15}\) for human prostatic phosphatase. Since we could not measure the concentration of unbound protein for technical reasons, a more accurate analysis involving Scatchard plots was not possible. A likely mechanism would involve three protein molecules with two Congo red molecules intercalated between them. The interaction of Congo red with protein molecules may involve a complex of multiple protein molecules with intercalated Congo red molecules. Intercalation as a mechanism of binding of Congo red molecules between peptide chains has also been suggested by Stopa and coworkers\(^\text{38}\).

**Oligomerization of proteins upon Congo red binding**

To test if Congo red binding involved oligomerization of protein molecules we added DTSSP a cleavable crosslinker to the protein solution in the absence and presence of Congo red. Analysis by crosslinking and non-reducing SDS-PAGE further confirmed the binding of Congo red to many proteins (Figure 7A). The control experiment in which the protein was crosslinked in the absence of Congo red revealed mostly monomeric species. The Congo red-bound protein bands showed up as red bands in the non-reducing gels until the gel was stained with Coomassie brilliant blue dye. This was due to the acidic conditions since Congo red is a pH indicator and turns blue at low pH. Upon drying and removal of acetic acid, the Congo red bands turned red again. The slower migration of the crosslinked Congo red-bound protein suggests that the proteins oligomerized during or after binding Congo red. Cleavage of the crosslinker under reducing conditions revealed only monomeric protein bands and free Congo red (Figure 7B). Free Congo red runs as a red band approximately the size of a 50 kD protein. Congo red has been
reported to self-associate and form ribbon-like micelles. It is possible that in the presence of sodium dodecyl sulfate, Congo red is a self-associated oligomer that runs as a high molecular weight species.

The results show that CR causes association of a variety of native proteins with different classes of secondary structure. The most reasonable explanation is that CR bridges two molecules by intercalating between hydrophobic surface patches with appropriate electrostatic regions for the sulfonate groups.

*Size and Shape of Congo red bound Oligomers*

Small angle scattering of X-rays by protein molecules can provide information about their size, shape and globularity. One of the most commonly used applications of SAXS is measurement of the size of a scattering molecule using Guinier analysis, which is based on the Gaussian shape of the scattering curve near zero angles. The Guinier plot for a homogeneous system is generally linear at small angles, allowing estimation of the radius of gyration of the particle, $R_g$. Figure 8A represents the Guinier plots for bovine \(\beta\)-lactoglobulin (250 \(\mu\)M) measured in the absence and presence of different Congo red concentrations (50 \(\mu\)M and 1 mM). All plots are linear functions, reflecting the fact that all three systems are essentially homogeneous, i.e. monodisperse. Dimensions of the protein are unaffected by the addition of small amounts of Congo red ($R_g=18.7 \pm 0.3$ and $18.7 \pm 0.3$ Å in the presence and the absence of 50 \(\mu\)M Congo red, respectively). However, in the presence of 4-fold excess of the dye over the protein, the $R_g$ value increases about three-fold ($R_g = 56.5 \pm 0.9$ Å), reflecting the Congo red-induced association of \(\beta\)-lactoglobulin. Additional information on the degree of protein association could be extracted from the analysis of the forward-scattered intensity values. The addition of 1 mM Congo red to
250 μM β-lactoglobulin results in a 28-fold increase in the I(0) value, indicating Congo red bound β-lactoglobulin forms large oligomers (i.e. ∼28mers).

The Kratky plot, I(S)x S² versus S, is a useful expression to describe the structural characteristics of a polymer. It has been shown that the shape of the Kratky plot is sensitive to protein conformation. In particular, for native globular proteins the Kratky plot has a characteristic position for the maximum that depends on the dimensions of the scattering particle, and shifts to smaller angles with increase in Rg. Figure 8 B represents Kratky plots for β-lactoglobulin in the absence and presence of two different concentrations of Congo red. Curves for β-lactoglobulin in the absence or the presence of 50 μM Congo red are superimposable and show the characteristic symmetric maximum of a native globular protein. However, the scattering profile for the protein in the presence of 1 mM Congo red (4-fold excess of protein) shows two very distinct maxima. The position of one of these maxima is comparable to that of the curves in the absence of Congo red, representing monomers. The very intense second maximum is observed at smaller angles, representing a specific large oligomer.

The hydrodynamic data allow us to estimate the shape of the β-lactoglobulin oligomer formed in the presence of 1 mM Congo red. The relationship between RS (Stokes radius) and Rg (radius of gyration) is quite sensitive to the shape and compactness of a protein (see Materials and Methods). For an ideal spherical particle Rg/R5=1.29, whereas for globular proteins the average value of this ratio is about 1.25. The Congo red-bound oligomeric form of β-lactoglobulin has Rg/Rs = 1.26, consistent with a spherical shape, rather than a linear polymer.
Birefringence assay in ex-vivo tissue sections

Congo red staining is a standard method used to examine ex-vivo tissue sections for amyloid fibril deposits. The ex-vivo tissue sections are tested for the presence of amyloid by first denaturing native proteins in the tissue sections followed by staining the fibrils with Congo red. These sections are then tested for birefringence under crossed polarization using light microscopy. The results described here show that Congo red binds to many native proteins and lacks secondary structure specificity. This would explain the false positive results obtained in tissue sections with cytoskeletal proteins that are stable under the conditions used for Congo red staining in tissue sections. This further confirms that specific crossed-β structure is not a requirement for Congo red binding, since cytoskeletal proteins have α, β, or coiled-coil structures. Our data showing Congo red binding to proteins from different secondary structure classes helps explain the false positive results obtained due to Congo red binding to cytoskeletal proteins. We suggest that caution is necessary when using Congo red as a method for testing for fibrils formed in vitro. Other dyes, such as thioflavin T, that are more specific to amyloid fibrils and do not bind native proteins, are better alternatives to Congo red for in vitro detection of fibrils.

Model of Congo red binding

Although Congo red is well established as an inhibitor of fibril formation for several proteins (e.g. Aβ, amylin, prions and insulin), there is considerable uncertainty as to the mechanism in which CR interacts with fibrils. In several cases evidence in support of an electrostatic interaction has been reported, however, in other cases it appears that specific interactions, and not simple electrostatic interactions are involved. The necessity for planarity has been
shown for the interaction with prion fibrils \(^{53}\). Since our results show that Congo red binds to native and partially-folded conformations of proteins, a likely mechanism of inhibition of amyloid fibril formation is that Congo red preferentially binds to the native or partially-folded states and stabilizes them (possibly as oligomers), thereby preventing formation of fibrils.

It has been reported that Congo red forms long rod-like “micelles” due to parallel stacking of the aromatics groups \(17\), and that these supramolecular forms of the dye specifically interact with \(\beta\)-sheets, due to the regular spacing of both assemblies. However, our results clearly show that this cannot be correct, since binding of CR to non-\(\beta\)-sheet proteins occurs, and, in fact, Congo red binds to native proteins from a wide variety of secondary structure classes. This suggests that specific secondary structural elements are not a requirement for binding of Congo red to proteins and amyloid fibrils. We have demonstrated, for the first time, oligomerization of proteins upon binding to Congo red, indicating that intercalation of dye molecules between multiple protein molecules leading to large oligomers is a probable mechanism of binding of Congo red to most native proteins. The shape of these oligomers was determined to be relatively spherical, indicating that the oligomerization does not lead to linear complexes. It is likely that Congo red, an elongated sulfonated hydrophobic molecule, binds to an exposed hydrophobic surface of the native or partially folded conformations, probably with specific complementary electrostatic interactions between charged side-chains and the sulfonate and amino groups of the dye, and induces association without concomitant structural changes. The complex of several protein molecules linked by intercalated Congo red molecules may remain as a soluble oligomer, as for IL-2 and \(\beta\)-lactoglobulin, or may become so large that it precipitates out of solution, as observed for lysozyme. It is most likely that both the hydrophobic and the electrostatic components of the structure of CR are critical for its binding to proteins.
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Figure Legends

Figure 1. Induced circular dichroism spectra of Congo red on binding to native or fibrillar proteins. A. Induced CD spectra on binding of 40 μM Congo red to 80 nM insulin fibrils (_____ ) and 400 nM Ig light chain fibrils ( - - - - ), and Congo red alone ( . . . . . ), in a 10 cm pathlength circular cell. Amyloid fibrils were made in vitro by stirring solutions of insulin and Ig light chain: the presence of fibrils was confirmed with EM or atomic force microscopy. B Induced CD spectra of 40 μM Congo red with 10 μM native insulin ( _____ ) or native Ig light chain ( - - - - ). Lower concentrations of fibrils were used to prevent formation of red precipitates.

Figure 2. Induced CD spectra of Congo red (40 μM) with citrate synthase (an all-α protein, ______ ), lysozyme (an α+β protein, __ ..__ ) and concavalin A (an all-β protein, - - - - ) under native conditions, 2 μM protein, 20 mM phosphate pH 7.5 and 150 mM NaCl, after 30 minutes incubation. The dotted line represents the spectra of unmixed solutions of 80 μM Congo red and 4 μM protein in a split cuvette.

Figure 3. Induced CD spectra for two right-handed β-helical proteins, pectate lyase ( __ .. __ ) and P22 tailspike protein ( - - - - ), and a left handed β-helical protein LpxA ( _____ ). Aggregated P22 tailspike protein ( . . . . . ) does not bind Congo red, and the resulting spectrum corresponds to that of Congo-red alone in the absence of protein.

Figure 4. FTIR spectra reveal that Congo red does not induce β-structure on binding to protein. A. ATR-FTIR spectra of IL-2 (an all-α-protein) showing the amide I and amide II regions with (- - - - ) and without Congo red ( _____ ). B. Lysozyme forms a red precipitate upon incubation
with Congo red (1 mg/ml lysozyme with 0.5 M Congo red), and the ATR-FTIR spectrum for this precipitate (- - - -) is compared to the spectrum for soluble native lysozyme ( _____ ).

**Figure 5.** Native Apomyoglobin shows an induced CD band upon binding to Congo red ( _____ ). Acidic unfolded apomyoglobin at pH 2 with no salt ( . . . . ) shows no CD bands. Acidic partially folded intermediates stabilized at pH 2 in the presence of salt ( ___ . . ___ ) and at pH 4 ( - - - - ) show induced CD bands that are different from the ones obtained from native apomyoglobin.

**Figure 6.** Titration of Congo red and β-lactoglobulin. **A.** Induced circular dichroism spectra of 40 µM Congo red increasing concentrations of β-lactoglobulin: 0.5 µM ( ____ ), 2.0 µM ( . . . . . ), 5.0 µM ( - - - - ), 10 µM (___ ___ ___) and 20 µM (___ __ ___). **B.** The ellipticity at 530 nm ( △ ) and 450 nm ( ○ ) plotted against β–lactoglobulin concentration and fitted to equation 2), indicating that 1.52 ± 0.05 molecules of Congo red bound per molecule of β-lactoglobulin.

**Figure 7.** Congo red induces oligomerization of native proteins. **A.** 8-25% non-reducing SDS-PAGE after crosslinking with DTSSP (a cleavable amine-reactive crosslinker), in the absence (-) and presence (+) of Congo red for pectate lyase, carbonic anhydrase (CA), β-lactoglobulin, IL-2 and lysozyme under non-reducing conditions. **B.** The same samples but after the crosslinker was cleaved by reducing the disulphide bond. Free Congo red (red bands) and monomeric protein bands (stained with Coomassie brilliant blue) appear.

**Figure 8.** Small-angle X-ray scattering analysis of the size of complexes of Congo red with β-lactoglobulin. **A.** Guinier Plots for 250 µM β-lactoglobulin alone (O), and in the presence of 50 µM ( V ), and 1 mM Congo red ( ). The increased slope at high CR concentration indicates a significantly larger R\textsubscript{g}. **B.** Kratky plots for β-lactoglobulin measured in the absence ( ____ ) and presence of two different concentrations of Congo red, 50 µM ( . . . . ) and 1
mM (___ ___). Both the plots demonstrate that the presence of Congo red (4 fold higher concentration than the protein) induces oligomerization of the β-lactoglobulin.
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