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Albumin-induced circular dichroism in Congo red: Applications for studies of amyloid-like fibril aggregates and binding sites

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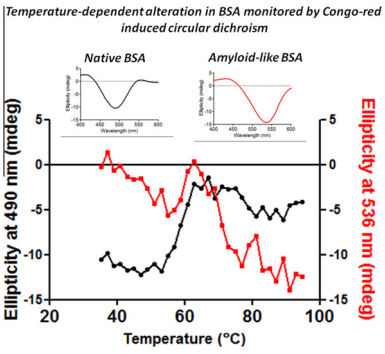
HIGHLIGHTS

- BSA induces chirality in Congo red.
- The induced circular dichroism spectrum of Congo red bound to native BSA is centred at 490 nm.
- The induced circular dichroism spectrum of Congo red bound to amyloid BSA is centred at 536 nm.
- The Congo red-ICD can be useful for monitoring the formation of amyloid aggregates.
- The Congo red-ICD can be useful for the classification of binding sites in BSA.

GRAPHICAL ABSTRACT



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ABSTRACT

Congo red (CR), one of the most commonly used dyes for the identification of amyloid fibril aggregates, is also a ligand of native bovine serum albumin (BSA). Induced circular dichroism (ICD) is a phenomenon observed when a chiral compound induces chirality in an achiral one. Here, we study the spectral properties and analytical applications of ICD in Congo red provoked by its interaction with BSA. The complex BSA:CR displays a strong ICD spectrum with a positive band at 412 nm and two negative bands at 356 and 490 nm. The use of site I and site II albumin ligands as warfarin and ibuprofen, respectively, provoked different alterations in the Congo red ICD spectrum. The BSA binding sites were modified by oxidation and the ICD signal was sensitive to this alteration. The thermal treatment of the BSA:CR complex (30–90 °C) was monitored by ICD at 490 nm and showed a sigmoidal pattern typical of phase transition in proteins. The altered ICD spectrum is consistent with the formation of amyloid-like fibril aggregates in BSA, which was confirmed by thioflavin T and Rayleigh scattering assays. In conclusion, the ICD provoked by the binding of Congo red to albumin may represent a new spectroscopic technique for studying alterations in the structure of albumin regarding its binding sites and the formation of amyloid aggregates.

Introduction

Amyloidosis is a systemic disorder characterised by the deposition of aggregates of misfolded proteins in the brain or peripheral tissues [1,2]. These protein aggregates are known as amyloid fibrils; these are highly organised, generally insoluble and rich in β-secondary structure [3]. Alzheimer's disease, Parkinson's disease, type 2 diabetes and cystic fibrosis are some examples of diseases where the deposition of amyloid fibrils is an important component in their pathogenesis [4–7]. The term amyloid has its origin in the discovery of these aggregates in biopsies and the use of iodine to stain this material. It is worth noting that iodine stains blue starch and its derivatives (*amylum* in Latin). Obviously, amyloid

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aggregates are not carbohydrate-based materials, but the name was still kept. Other hallmarks in the characterisation of deposits of amyloid fibrils in tissues was the identification of a birefringent effect when these protein aggregates were stained by Congo red and examined by polarised light microscopy, which became the gold standard test for histological confirmation of amyloidosis [[8] and references therein].

Serum albumin is the major protein in body fluids. From a pharmacological perspective, albumin acts as a carrier of drugs in the systemic circulation, hence developing an important role in the drug pharmacokinetics [9]. For that, albumin has two main binding sites, referred to as site I and site II, which are located in the hydrophobic cavities of the subdomains IIA and IIIA, respectively [10,11]. Albumin is also frequently used as a prototype protein for studying the physical and chemical events that underlie the formation of amyloid fibril aggregates [12,13]. In these studies, the formation of soluble amyloid aggregates is usually evidenced by a red-shift in the absorption of Congo red [14], enhanced thioflavin T intrinsic fluorescence [15], Rayleigh scattering caused by the protein aggregation and the direct visualisation of the fibrils by transmission electronic microscopy [16].

Another interesting property of Congo red is its susceptibility to acquire chirality when bound to some proteins. This phenomenon is also named induced circular dichroism (ICD) and results from the attachment of the optically inactive substance inside the asymmetric microenvironment, which forms the protein binding sites [17]. Among the proteins for which ICD in Congo red has been demonstrated are citrate synthase, lysozyme, concanavalin A and poly-L-lysine [18,19].

Considering the wide applicability of Congo red for characterisation of amyloid fibrils, here, we performed a comprehensive study of the spectroscopic characteristics of the induced circular dichroism in Congo red provoked by its binding in native and amyloid forms of bovine serum albumin. By following the temperature-dependent alteration in the ICD spectrum, it was possible to observe, for the first time, the phase transition from native to amyloid form using the ICD technique. This experimental protocol can be very useful for those interested in the study of amyloid fibril inhibitors.

Materials and methods

Chemicals and solutions

Bovine serum albumin (BSA), sodium salt of benzidinedia zo-bis-1-naphtylamine-4-sulphonic acid (Congo red), thioflavin T, phenylbutazone, warfarin, naproxen, ibuprofen, dansylamide, dansylglycine, sodium dodecyl sulphate (SDS) and taurine were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Stock solutions of the pharmaceuticals (10 mM) were prepared in ethyl alcohol. Stock solutions of dansylglycine (5 mM) were prepared in 10 mM hydrochloric acid. Stock solutions of dansylamide (5 mM) were prepared in dimethyl sulphoxide. Stock solutions of Congo red (10 mM) were prepared in 50 mM phosphate buffer at pH 7.0. BSA was dissolved in 50 mM phosphate buffer at pH 7.0 to give a 1 mM stock solution, which was stored at 4 °C. Protein concentration was determined by measuring its absorbance at 280 nm (ε_{280nm} = 43,890 M^{-1} cm^{-1}) [20]. The concentration of the 5% commercial solution of hypochlorous acid (HOCl) was determined by measuring its absorbance at 292 nm in 0.01 M NaOH (ε_{292nm} = 350 M^{-1} cm^{-1}) [21]. The stock solutions of HOCl (100 mM) were prepared in water. Stock solutions of hypobromous acid (HOBr) 100 mM were synthesised by combining 100 mM HOCl and 200 mM NaBr in water [22]. Stock solutions of taurine bromamine (Tau–NHBr) were prepared immediately prior to use by the addition of 5 mM HOBr to 500 mM taurine in 10 mM phosphate buffer at pH 7.0 [23]. The concentrations of Tau–NHBr were determined spectrophotometrically (ε_{288nm} = 430 M^{-1} cm^{-1}) [23]. The UV–Vis experiments were performed using a Perkin Elmer Lambda 35 UV–Vis spectrophotometer (Shelton, CT, USA).

Determination of binding sites: displacement experiments

Determination of the binding sites of Congo red in BSA was performed using the fluorescent probes dansylamide for site I [24] and dansylglycine for site II [25]. The spectrofluorimeter was adjusted to the following parameters: excitation at 340 nm and emission in the range 400–650 nm, slit widths of 2.5 nm for excitation and 10 nm for emission wavelengths, total volume 3 mL in a quartz cuvette with a 10 mm path length and magnetic stirring. The experiments were performed by the addition of varying amounts of Congo red (0–5 μM, final concentrations) to a mixture of 5 μM BSA and 5 μM dansylamide or dansylglycine in 50 mM phosphate buffer at pH 7.0. After the addition of Congo red, the mixtures were incubated for 5 min at 25 °C before the measurements were taken. The fluorescence spectra were obtained using a Perkin Elmer LS 55 spectrofluorimeter (Shelton, CT, USA) [25].

Circular dichroism experiments: intrinsic and induced circular dichroism

The intrinsic circular dichroism spectra of BSA were obtained with 1 nm step resolution, response time of 1 s and scanning speed of 50 nm/min in the range of 195–250 nm (far-UV-CD). Mixtures of 30 μM BSA and 30 μM Congo red in 50 mM phosphate buffer at pH 7.0 were incubated for 1 h at 25 °C. Then, the mixtures were 30-fold diluted and the far-UV-CD spectra recorded in a 2 mm path length quartz cuvette. The baseline (50 mM phosphate buffer) was subtracted from all measurements.

The thermal denaturation curves were determined by monitoring the change in ellipticity at 222 nm in the temperature range 25–95 °C with a heating rate of 1 °C/min. The temperature was measured at 1 °C intervals, holding for 30 s before the measurements at each temperature.

The induced circular dichroism (ICD) spectra were obtained with 1 nm step resolution, response time of 1 s and scanning speed of 50 nm/min in the range of 250–600 nm (near-UV-CD). The measurements were performed using 30 μM BSA in the presence or absence of 30 μM Congo red at 25 °C. A 3 mL quartz cuvette with a 10 mm path length under magnetic stirring was used for the measurements. The baseline (50 mM phosphate buffer) was subtracted from all measurements.

The displacement of Congo red from BSA, measured by the loss of the ICD signal, was performed by the addition of varying amounts of the ligands (0–120 μM, final concentrations) to a mixture of 30 μM BSA and 30 μM Congo red in 50 mM phosphate buffer at pH 7.0. After the addition of ligands, the mixtures were incubated for 5 min at 25 °C before the ICD measurements. The ligands used were phenylbutazone [26] and warfarin [27] for site I, and ibuprofen [28], naproxen [29] and dansylglycine [25] for site II.

The temperature-induced alteration of the ICD signal of Congo red was monitored by scanning the spectra (350–600 nm) in the temperature range 30–90 °C with a heating rate of 1 °C/min, holding for 30 s before the measurements and scanning at 2 °C intervals. The experiments were performed using a Jasco J-815 spectropolarimeter equipped with a thermostatically controlled cell holder (Jasco, Tokyo, Japan).

Oxidation of BSA

Stock solutions of HOCl, HOBr and Tau-NHBr were prepared as described above. The reaction mixtures contained 30 μM BSA and