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SPECTRAL BEHAVIOR OF AMYLOID-SPECIFIC DYES IN PROTEIN-LIPID SYSTEMS. III. CONGO RED INTERACTIONS WITH NATIVE PROTEINS

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A number of so-called conformational diseases (Parkinson's, Alzheimer's and Huntington's diseases, type II diabetes, spongiform encephalopathies, systemic amyloidosis) are associated with the deposition in various tissues highly-ordered protein aggregates (amyloid fibrils) that kill cells or prevent them from functioning properly. Amyloid fibrils are organized in a cross β-structure with a helical array of β-sheets, in which the long axis of the fibril is parallel to the long axis of the helix and is perpendicular to the β-strands Amyloid can be identified using a range of techniques: electron and atomic force microscopy, X-ray fibril diffraction, thioflavin T fluorescence, Congo Red (CR) birefringence or spectrophotometric assay. However, therapeutic detection of amyloid fibrils with CR test may be hampered by CR ability to form complexes with native proteins. In the present study we investigated CR binding to a series of native proteins – hemoglobin (Hb), cytochrome c (cyt c), ribonuclease A (RNase), human serum albumin (HSA). CR interaction with Hb and cyt c was followed by absorbance decrease and long wavelength shift of spectrum maximum in the case of Hb, indicating that native protein structure contains binding sites for CR. Association constant (K_b) and binding stoichiometry (n) recovered from the data analysis within the framework of Langmuir adsorption model were found to be: $K_b=(2.1 \pm 0.3)\times10^5$ M^{-1}, $n=3.3 \pm 0.5$ for Hb and $K_b=(6.0 \pm 0.9)\times10^4$ M^{-1}, $n=1.0 \pm 0.3$ for cyt *c*. The presence of lipid vesicles composed of phosphatidylcholine and cardiolipin did not exert influence on CR-Hb interactions. In contrast, association constant for CR-cyt *c* complexation markedly increased. This finding was interpreted in terms of cyt *c* unfolding at lipid-water interface coupled with exposure of additional CR binding sites on the protein surface. Formation of CR complexes with RNase and HSA was followed by the long-wavelength shift of absorption maxima. CR-HSA binding curves have Langmuir-like shape, whereas CR-RNase adsorption isotherms are slightly sigmoidal pointing to cooperative nature of the binding process. The binding parameters were estimated to be $K_b=(1.3 \pm 0.3)\times10^4$ M^{-1}, $n=2.3 \pm 0.5$ for HAS and $K_b=(3.4 \pm 0.3)\times10^4$ M^{-1}, $n=0.6 \pm 0.1$ and Hill parameter $\alpha= 1.1\pm0.2$ for RNase.

KEY WORDS : Congo Red, hemoglobin, ribonuclease, cytochrome *c*, albumin, liposomes, protein-dye complexes

Amyloid is a general term for protein deposits in tissues that share at least two characteristics: (1) extracellular, nonbranching fibrils, (2) cross-beta sheet quaternary structure that creates a characteristic X-ray diffraction pattern. Amyloid-like fibrils are elongated, insoluble protein aggregates, formed in vivo in association with a number of so-called conformational diseases or in vitro from soluble native proteins under strongly denaturing conditions. It is still not known whether amyloid plaques are a cause or symptom of diseases like systemic amyloidosis; diabetes mellitus type 2; Alzheimer's, Parkinson's, or Huntington's disease; and spongiform encephalopathies (prion diseases). Amyloid fibrils are organized in a cross β-structure with a helical array of β-sheets, in which the long axis of the fibril is parallel to the long axis of the helix and is perpendicular to the β-strands [1-3]. The ordered fibrillar aggregates have been studied by a range of techniques: transmission electron microscopy, atomic force microscopy, X-ray fibril diffraction, thioflavin T fluorescence, Congo Red (CR) birefringence or spectrophotometric assay. Congo Red alone has an absorbance spectrum with a maximum at 487 nm. In the presence of amyloid fibrils CR absorbance increases and the absorption maximum shifts to 505 nm. These spectral changes are indicative of amyloid fibril formation [4]. However, a wide variety of native proteins

exhibited similar alterations in CR absorption spectra pointing to that CR can form complexes with nonaggregated protein species [5]. This process can hamper detection of amyloid fibrils with CR test. In view of this in the present work we have centered our attention on the study of CR interaction with four well-characterized proteins – hemoglobin (Hb), cytochrome c (cyt c), ribonuclease A (RNase), albumin (HSA). Our goal was twofold. First, it seemed of importance to ascertain whether CR binding to Hb and cyt c is influenced by the presence of lipid-water interfaces. Second, it was of interest to analyze the differences in CR association with native and denaturated proteins (HAS, RNase). Clarifying these issues is of significance for correct detection of amyloid fibrils in different biological samples.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine (PC) and beef heart cardiolipin (CL) were purchased from Biolek (Kharkiv, Ukraine). Congo Red was from Aldrich (Milwaukee WI). Horse hemoglobin, RNase, HAS were purchased from Reanal (Hungary). Butylated hydroxytoluene (BHT) was from Merck (Germany). All chemicals were of analytical grade. Absorption measurements with CR were performed using SF-46 spectrophotometer. CR, Hb, RNase, cyt c HSA concentrations were determined spectrophotometrically using the extinction coefficients $\varepsilon_{CR}^{498} = 3.7 \times 10^4$ M-1cm-1, $\varepsilon_{Hb}^{406} = 1.415 \times 10^5$ M-1cm-1 per heme, $\varepsilon_{RNase}^{280} = 1.4 \times 10^4$ M-1cm-1, $\varepsilon_{Cytc}^{406} = 1.05 \times 10^5$ M-1cm-1 per heme and $\varepsilon_{HSA}^{278} = 4.25 \times 10^4$ M-1cm-1, respectively.

Large unilamellar lipid vesicles composed of PC and its mixture with 10 mol% CL were prepared by the extrusion technique. The thin lipid films were obtained by evaporation of lipids ethanol solutions and then hydrated with 1.2 ml of 5 mM sodium-phosphate buffer (pH 7.3). Lipid suspension was then extruded through a 100 nm pore size polycarbonate filter. Phospholipid concentration was determined according to the procedure of Bartlett [6]. RNase and HSA were denaturated by incubating protein solutions for 24 hr, pH 7.4, 70 ˚C.

CR is a long thin and flat molecule having two negatively charged sulfonic acid groups.

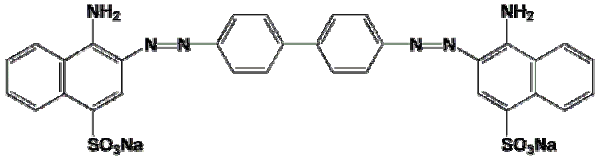

Fig. 1. Structure of CR molecule.

Quantitative parameters of CR binding to protein-lipid complexes were determined by analyzing the dependencies of dye absorbance changes on CR concentration. It was assumed that the observed absorbance increase (at 500 nm) is proportional to the concentration of bound dye:

$$\Delta A = aB, \quad (1)$$

where a is a coefficient of proportionality. If one protein molecule contains n CR binding sites in terms of the Langmuir's model association constant (K_b) can be written as:

$$K_b = \frac{B}{F(nP - B)} = \frac{B}{(Z_0 - B)(nP - B)}, \quad (2)$$

where P is the total protein concentration, F is the concentration of free dye, Z_0 is total dye concentration. Accordingly, Eqs. (1) and (2) can be rearranged to give:

$$\Delta A = 0.5a\left(Z_0 + nP + \frac{1}{K_b} - \sqrt{\left(Z_0 + nP + \frac{1}{K_b}\right)^2 - 4Z_0 nP}\right) \quad (3)$$

In the case where the dye-protein binding exhibited the feature of cooperative process dye-protein binding can also occur via cooperative binding. In this case Eq. (1) takes the form:

$$\Delta A = aPn\frac{(Z_0 - B)^{\alpha}}{\frac{1}{K_b^{\alpha}} + (Z_0 - B)^{\alpha}} \quad (4)$$

where α is the Hill parameter. The binding parameters (K_b and n) were derived from the fitting procedure involving minimization of the function:

$$f = \frac{1}{N}\sum_{i=1}^{N}\left(\Delta A_{\exp} - \Delta A_t\right)^2 \quad (5)$$

where $\Delta A_{\exp}$ is experimental ΔA value, ΔA_t is ΔA calculated from Eq. (3) or Eq (4), N is the number of experimental points in ΔA dependency on protein concentration.

RESULTS AND DISCUSSION

CR association with hemoglobin and cytochrome c in the presence of lipid-water interface

A lot of protein are functioning in membrane-bound state. Accordingly, membrane complexation may affect amyloidogenic properties of the proteins. Therefore, it is important to clarify how CR binding to lipids and lipid-protein interactions may influence protein-associating ability of this dye. At the first step of the study we investigated the dye binding to heme-containing proteins, Hb and cyt *c*. These proteins avidly associate with lipid bilayers via electrostatic and hydrophobic interactions whose relative contributions are different for Hb and cyt *c*. Hb-lipid binding occurs mainly via hydrophobic interactions while cyt *c*-lipid complexes are stabilized predominantly by electrostatic forces. In both cases protein-membrane association is accompanied by the changes in protein conformational state and lipid-bilayer structure. Hb penetrates into hydrophobic membrane region and causes lipid packing disorder [7]. Cyt *c* initiates domain formation in lipid bilayers, and modifies membrane structure and dynamics [8-9]. Hb and cyt *c* molecules undergo unfolding on lipid surface, which can be followed by the heme loss [9-10].Lipid-induced conformational changes of the proteins bear resemblance to denaturation process. In this way lipids may exert influence on CR-associating ability of the proteins.

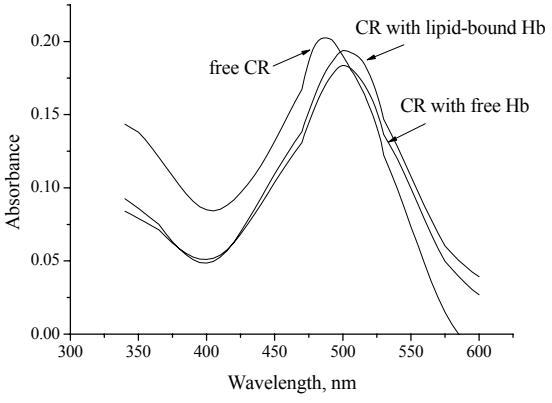

Fig. 1. CR absorption spectra in buffer, Hb and Hb-lipid mixtures

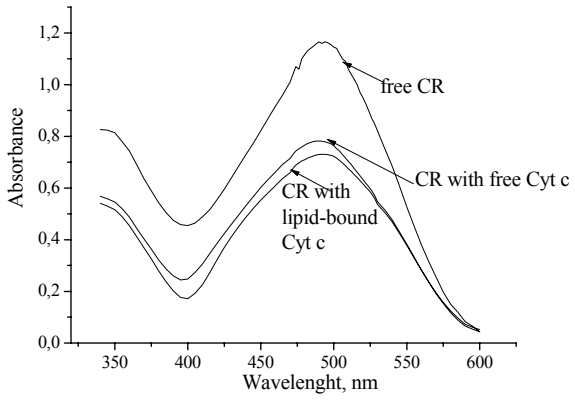

Fig. 2. CR absorption spectra in buffer, cyt *c* and cyt *c*-lipid mixtures

As shown in Fig. 1, CR-Hb interactions is followed by the absorbance decrease and shift of spectrum maximum from 487 nm to 504 nm. Such absorbance shift is commonly regarded as indicative of the presence of amyloid fibrils [4], however, our data [11] and the results of other authors [5] suggest that CR is capable of forming complexes with native

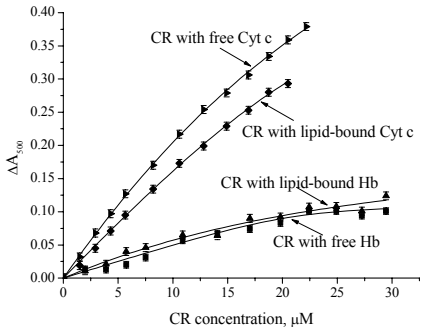

Fig. 3. The isotherm of CR binding to Hb and cyt *c* in the presence and absence of model memranes

proteins. The pronounced red shift of maximum position is indicative of the chromophore transfer into less polar environment. Hb surface contains considerable amount of hydrophobic and positively charged groups, which may serve as binding sites for CR molecule [11]. After addition of lipid vesicles to Hb solution the dye spectra remain virtually unchanged (Fig. 1). CR complexation with cyt *c* was accompanied by absorbance decrease, but spectral maximum remained around 490 nm. It seems likely, cyt *c*-CR binding is governed by electrostatic interactions between CR sulfonic groups and positively charged amino acid residues on the protein surface. Similar to Hb case, addition of liposomes did not cause appreciable CR spectral changes. Previously it was demonstrated that CR does not associate with PC/CL model membranes [12]. For this reason, lipid addition does not lead to appearance of new binding sites for CR molecules.

Table 1

Association constant and stoichiometry of Congo Red binding to Hb and cyt *c*

System	*n*	K_b, M^{-1}
CR + Hb	3.3 ± 0.5	$(2.1 \pm 0.3)\times10^5$
CR + Hb + PC/CL liposomes	3.0 ± 0.5	$(2.3 \pm 0.3)\times10^5$
CR + cyt *c*	1.0 ± 0.3	$(6.0 \pm 0.9)\times10^4$
CR + cyt *c*+ PC/CL liposomes	1.2 ± 0.2	$(3.6 \pm 0.5)\times10^5$

To better understand the nature of dye-protein interactions the data obtained were analyzed quantitatively in terms of the above adsorption model (Eq. 3). Analysis of the recovered binding parameters presented in Table 1 shows that addition of model membranes does not influence binding stoichiometry and CR affinity for Hb, either free in solution or bound to lipids. In contrast, in cyt *c*-lipid systems K_b value was increased by about 6 times. As indicated above, cyt *c* undergoes experiences unfolding when bound to negatively charged lipid bilayer [9]. It cannot be excluded that such structural changes of the protein molecule are accompanied by the exposure of additional binding site for CR.

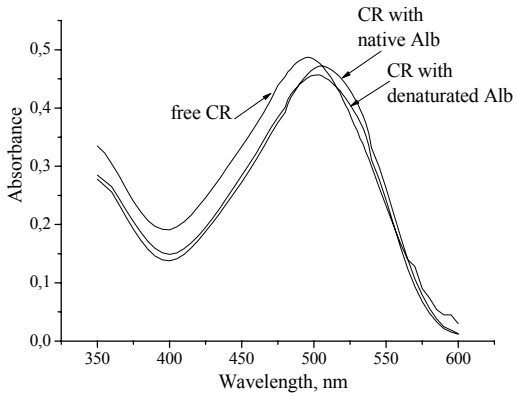

Fig. 4. CR absorption spectra in buffer and in solutions containing native and denaturated albumin

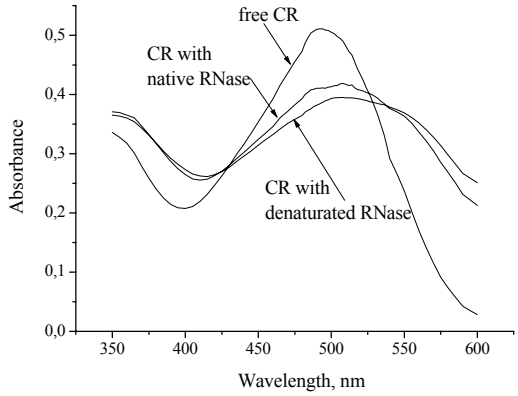

Fig. 5. CR absorption spectra in buffer and in solutions containing native and denaturated ribonuclease

CR binding to albumin and ribonuclease A

At the next step of the study we investigated CR interactions with two functionally relevant proteins – ribonuclease A and human serum albumin. RNase is type of a nuclease that catalyzes the degradation of RNA into smaller components. HSA is the most abundant blood plasma protein that is produced in the liver and forms a large proportion of all plasma protein. HSA normally constitutes about 70% of human plasma proteins. Its main function is recruitment and transport of some metabolits, for example fatty acids and bilirubin.

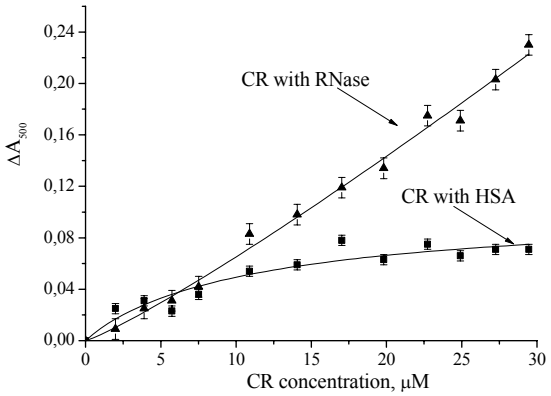

Fig. 6. The isotherm of CR binding to HSA and RNase

Formation of CR complexes with both native and denaturated RNase and HSA was followed by the long-wavelength shift (about 10 nm) of absorption maxima originating presumably from lowered polarity of chromophore's microenvironment (Fig. 4, 5). In the case of HSA absorbance decrease was less than in the case of RNase. As seen in Fig. 4, isotherm of CR binding to HSA is hyperbolic in shape, while that for RNase in featured by slightly sigmoidal. Therefore, the binding data obtained for HSA and RNase were approximated by the Langmuir (Eq. (3)) and Hill (Eq. (4)) adsorption models, respectively. The recovered binding parameters presented in Table 2 indicate that CR complexation with RNase, in contrast to HSA, displays slight cooperativity. This may be a consequence of aggregation of negatively charged the dye anions on the surface of positively charged RNase.

Table 2

Parameters of Congo Red association with RNase and HSA

System	n	K_b, M^{-1}	α
CR + RNase	0.6 ± 0.1	$(3.4 \pm 0.3)\times10^4$	1.1 ± 0.2
CR + HSA	2.3 ± 0.5	$(1.3 \pm 0.3)\times10^4$	-

CR interaction with denaturated proteins results in negligible decrease of the dye absorbance without maximum shift. Probably, the employed denaturing conditions are insufficient for amyloid fibril formation. CR binding to denatured RNase was found to be followed by the protein precipitation. This finding can be explained by the fact that CR *per se* has been reported to cause protein aggregation not associated with fibril formation [4]. Apparently, attractive electrostatic interactions between negatively charged CR and cationic RNase partly neutralize protein charge thereby creating conditions which favor protein-protein interactions.

CONCLUSIONS

In the experiment with a series of water soluble proteins hemoglobin, cytochrome *c*, ribonuclease A and human serum albumin it was demonstrated that amyloid-specific dye Congo Red is featured by pronounced ability to form complexes with native proteins stabilized presumably by both electrostatic and hydrophobic interactions. Protein-lipid association does not affect CR-Hb binding but provokes appearance of new binding sites for the dye on cyt *c* surface. CR-RNase complexation displays slight cooperativity. Heat

denaturation of HSA and RNase was not followed by CR spectral changes characteristic of protein fibrillization.

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