БІОФІЗИКА КЛІТИНИ

УДК 577.37

SPECTRAL BEHAVIOR OF AMYLOID-SPECIFIC DYES IN PROTEIN-LIPID SYSTEMS. II. CONGO RED INTERACTIONS WITH HEMOGLOBIN

O.K. Zakharenko, G.P. Gorbenko, V.M. Trusova, A.V. Finashin
V.N. Karazin Kharkov National University, 4 Svobody Sq., Kharkov, 61077
Received 7 May, 2008

A number of so-called conformational diseases including neurological disorders (Parkinson's, Alzheimer's and Huntington's diseases), type II diabetes, spongiform encephalopathies, systemic amyloidosis, *etc*., are associated with the deposition in tissue of highly ordered aggregates of specific proteins. Amyloid fibrils are usually detected by several techniques including Thioflavin T fluorescence, Congo Red (CR) birefringence of spectrophotometric assay, electron microscopy, *etc*. However, application of amyloid-specific agents such as CR to amyloid detection may be hampered by the dye ability to associate not only with fibrillar structures but also with monomeric protein species. In view of this reasoning the present study was directed toward the examination of the interactions between CR and hemoglobin (Hb), the protein with well-characterized structure and physicochemical properties. The binding of CR to native and denaturated Hb was studied using absorption spectroscopy technique. Differential absorption spectrum of CR associated with denaturated protein was found to exhibit maximum close to that characteristic of fibrillar structures (545 nm), thereby providing arguments in favor of Hb fibrillization. Formation of CR complexes with native Hb was followed by the long-wavelength shift (~10 nm) of absorption maxima being indicative of the probe transfer to the environment of lower polarity. Based on analysis of Hb crystal structure the tentative location of CR in the protein molecule has been identified. The most probable dye binding site was assumed to involve the hydrophobic cavity between Lys16 and Lys60 serving as anchors for two negatively charged CR sulfonic groups. Quantitative parameters of CR complexation with native Hb – association constant (K_b) and number of binding sites (n) – were determined by analyzing the dependencies of dye absorbance changes upon varying protein concentration. Approximation of experimental dependencies by Langmuir binding model yielded the values of K_b and n *ca.* 2.6×10^5 M^{-1} and 1.4, respectively. For thermally denaturated Hb, the shape of CR binding curve was revealed to change from Langmuir-like to sigmoidal. Simulation results showed that such a behavior of binding curve is characteristic of preferential dye association with aggregated protein species.

KEY WORDS: Congo Red, hemoglobin, protein-dye complexes

Protein misfolding followed by the formation of highly-ordered aggregates (termed amyloids) is currently considered as a direct cause of a large group of diseases, such as Parkinson's, Alzheimer's and Huntington's, type II diabetes, spongiform encephalopathies, systemic amyloidosis, etc. Amyloid fibrils represent a polymeric form of a protein whose internal structure is largely a cross-β-sheet with β-strands running perpendicular to the long axis of the fibrils [1-3]. In vitro, fibrillization-favoring conditions are created by lowering pH, elevating temperature, adding organic solvents or denaturants, *etc.*, while in vivo, abnormal partial unfolding or folding may arise from mutations, oxidative or heat stress or destabilization of the protein structure upon its adsorption at interfaces. The fact that amyloid fibrils can be self-assembled from a wide range of different polypeptides and proteins led to an increasing belief that the ability to form amyloid fibrils is a generic property of polypeptide chain with cross-β structure being a universal energetic minimum for aggregated protein [4,5]. Amyloid fibrils are usually detected by several techniques: Thioflavin T (ThT) fluorescence, Congo Red (CR) birefringence of spectrophotometric assay, electron microscopy and FTIR [1]. Originally amyloid fibrils have been identified with CR staining of histological samples. While employing amyloid-specific agents such as ThT or CR one should bear in mind that these dyes may form complexes not only with fibrillar structures but also with monomeric

protein species. Clearly, the implications of such a property should be evaluated in each system under investigation. In view of this, the present study was focused on examining the interactions between CR and hemoglobin (Hb). Due to well-characterized structure and physicochemical properties, Hb can be used for elucidating general features of CR interactions with proteins.

MATERIALS AND METHODS

Congo red was from Aldrich (Milwaukee WI). Horse hemoglobin was purchased from Reanal (Hungary). All chemicals were of analytical grade. Absorption measurements with CR were performed using SF-46 spectrophotometer. CR and Hb concentrations were determined spectrophotometrically using the extinction coefficients $\varepsilon_{CR}^{498} = 3.7 \times 10^4\ \mathrm{M^{-1}cm^{-1}}$ and $\varepsilon_{Hb}^{406} = 1.5 \times 10^5\ \mathrm{M^{-1}cm^{-1}}$, respectively. The experimental conditions used for Hb denaturation involved: **(1)** 20 day incubation at pH 3 and 25 ˚C, and **(2)** 24 h incubation at pH 7.4 and 70 ˚C. Analysis of Hb crystal structure was performed by means of WebLab ViewerPro Trial37 software.

CR is a long thin and flat molecule having two negatively charged sulfonic acid groups [5].

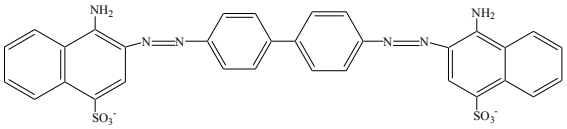

Fig. 1. Structure of CR molecule.

Quantitative parameters of CR binding to Hb were determined by analyzing the dependencies of dye absorbance changes upon varying protein concentration. It was assumed that the observed absorbance increase (at 540 nm) is proportional to the concentration of bound protein:

$$\Delta A = aB, \tag{1}$$

where a is a coefficient of proportionality. If one protein molecule contains n CR binding sites, the association constant (K_b) can be written as:

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

where P is the total protein concentration, F is the concentration of free dye, Z_0 is total dye concentration.

Accordingly, Eq. (1) can be rearranged to give:

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

The binding parameters (K_b and n) were derived by the fitting procedure involving minimization of the function:

$$f = \frac{1}{N}\sum_{i=1}^{N}\left(\Delta A_{\exp} - \Delta A_t\right)^2 \tag{4}$$

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

RESULTS AND DISCUSSION

CR specific binding to amyloid fibers manifests itself in the yellow-green birefringence and characteristic peak at 545 nm in the differential absorption spectra. Based on crystallographic data CR has been suggested to orient parallel to the fibril axis [6]. Native β-sheet would need to contain at least 6 strands to accommodate CR molecule whose length is *ca.* 21 Å [5]. At the first step of the study we made an attempt to prepare fibrillar Hb samples

by the protein subjection to harsh denaturing conditions (pH 3, incubation up to 20 days at 25 ˚C). Fig. 1 shows CR differential absorption spectra for the native and denaturated (under conditions **(1)**) protein.

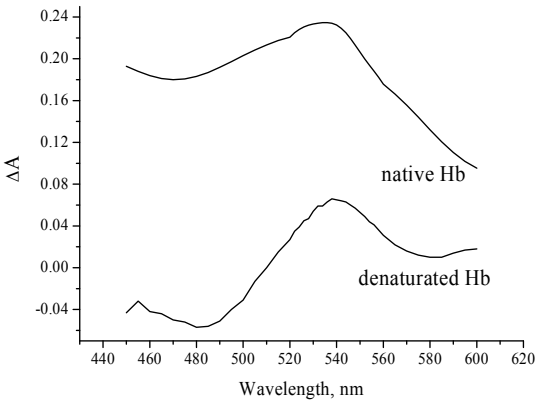

Fig. 1. CR differential spectra. Protein concentration was 2.5 μM (denaturing conditions **(1)**)

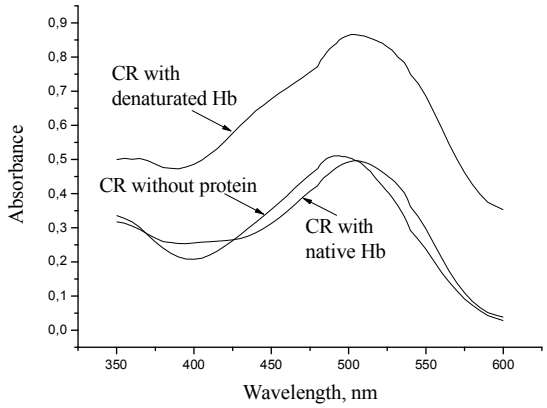

Fig. 2. CR absorption spectra (denaturing conditions **(2)**)

Differential absorption spectrum of CR associated with denaturated protein exhibits maximum close to that characteristic of fibrillar structures (545 nm), thereby providing arguments in favor of Hb fibrillization. However, similar, although less defined, maximum position was observed for native Hb. Next, we examined CR interactions with native Hb in more detail. As shown in Fig. 2, Hb addition to CR solution was followed by the long-wavelength shift (about 10 nm) of absorption maxima and increase of the dye absorbance (presumably, due to light scattering) for denatured Hb. The pronounced red shift of maximum position is indicative of the chromophore transfer into less polar environment. CR has several functional groups that can potentially interact with native proteins or amyloid fibrils by different mechanisms including i) H-bonding with amino acid groups, ii) ionic and hydrophobic interactions, iii) steric intercalation of the dye between β-sheets [1]. Taking into account the size of CR molecule and the fact that distance between negatively charged sulfonic groups is ~ 19.3 Å, we tried to identify tentative CR binding site in Hb molecule through analysis of the protein crystal structure, assuming that positively charged Lys residues can serve as anchors for CR sulfonic groups. Found in such a way the most probable CR binding site involves hydrophobic cavity between Lys16 and Lys60 separated by a distance 19.8 Å (Fig. 3). To obtain quantitative information on CR-Hb complexation we analyzed $\Delta A(P)$ dependencies (Fig. 4) in terms of the above model (Eqs (1)-(4)). For native Hb the binding parameters were found to be: $K_b \approx 2.6\times10^5\,\mathrm{M}^{-1}$, $n \approx 1.4$. Within the concentration range employed in our experiments tetrameric Hb molecule tends to dissociate into αβ-dimers, whose α-subunit contains one binding site for CR.

After Hb denaturation under conditions **(2)**, the binding curve proved to change its shape from Langmuir-like to sigmoidal (Fig. 5). To explain this finding, we assumed that there exist two types of CR binding sites, located at monomeric and aggregated protein species, respectively. Degree of oligomerization increases with total protein concentration, so that the number of the centers of different types appears to be dependent on *P*. In the simplest way, such dependence can be described in the following manner:

$$N_1(P) = n_1 P^{\beta+1}; \qquad N_2(P) = n_2 P\left(1 - P^{\beta}\right) \tag{5}$$

where N_1 is the number of CR binding sites with protein monomers, and N_2 is the number of CR binding sites with protein aggregates, β is a parameter related to oligomerization degree.

If $\beta = 0$, the protein is fully monomeric, while at increasing $\beta < 0$ monomeric species coexist with aggregated ones.

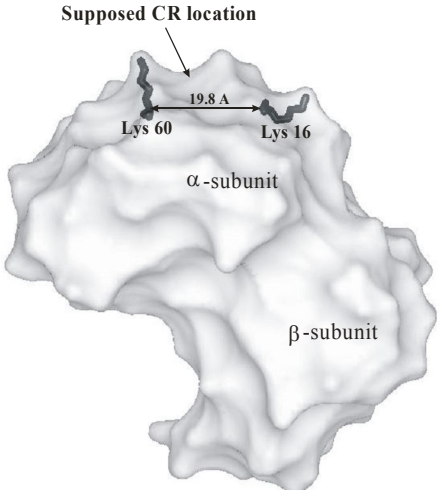

Fig. 3. Schematic representation of CR location in Hb

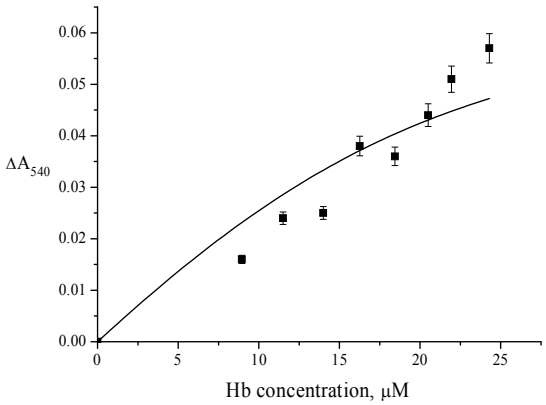

Fig. 4. The isotherm of CR binding to native Hb

Shown in Fig. 6 are simulation results illustrating that the binding curve has sigmoidal shape when CR binds much stronger with aggregates compared to monomers ($K_{b2} >> K_{b1}$).

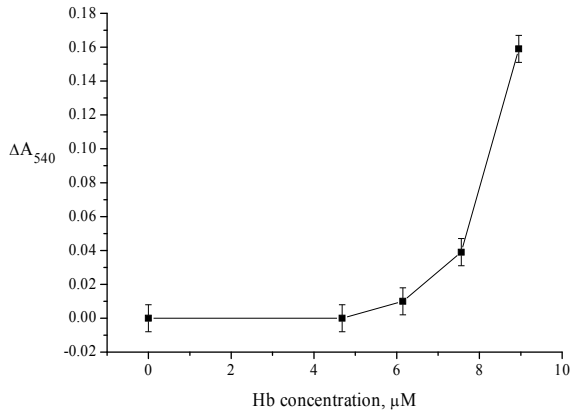

Fig. 5. The isotherm of CR binding to denaturated Hb

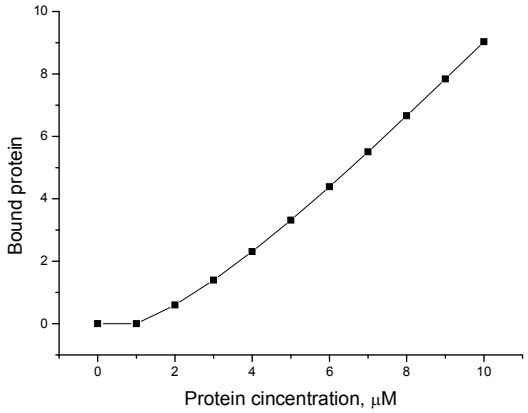

Fig. 6. Theoretical binding curve

CONCLUSIONS

The binding mechanism of amyloid-specific dye CR to native and denaturated protein in terms of binding affinity and stoichiometry was quantitated from the absorption spectroscopy. For native protein association constant and the number of binding sites were determined to be 2.6×10^5 M^{-1} and 1.4, respectively. Thermal denaturation of the protein gives rise to the alterations of CR-Hb binding curve from Langmuir-like to sigmoidal. Simulation results revealed that such a behavior of binding curve is characteristic of preferential dye association with aggregated protein species.

REFERENCES

1. M.R. Nilsson // Methods. 2004. 34. P. 151-160.
2. M.R.H. Krebs, E.H.C. Bromley, A.M. Donald // J. Struct. Biol. 2005. 149. P. 30-37.
3. L.C. Serpell // Biochim. Biophys. Acta. 2000. V. 1502. P. 16–30.
4. O.S. Makin // Proc. Natl. Acad. Sci. 2005. V. 102. P. 315-320.
5. V. Uversky and A. Fink // Biochim. Biophys. Acta. 2004. V. 1698. P. 131-153.
6. D.B. Carter and K.C. Chou // Neurobiol. Aging. 1998. V. 19. P. 37-40.