Acta Biochimica Polonica

Vol. 53 No. 1/2006, 87–91

on-line at: www.actabp.pl

Regular paper

Congo red interaction with α-proteins⋆

Jolanta Sereikaite✉ and Vladas-Algirdas Bumelis

Department of Chemistry and Bioengineering, Faculty of Fundamental Sciences, Vilnius Gediminas Technical University, Vilnius, Lithuania; ✉e-mail: sjolanta@fm.vtu.lt

Received: 06 November, 2005; revised: 07 March, 2006; accepted: 12 March, 2006
available on-line: 15 March, 2006

The ability of Congo red to form complexes with α-proteins, human growth hormone and human interferon-α2b, was found by absorption difference spectroscopy. A human growth hormone–Congo red complex was isolated by gel-permeation chromatography, and its visible absorption spectrum was registered in comparison to free dye. The ability of Congo red to induce dimerization of human growth hormone was demonstrated using chemical cross-linking agents 1,3,5-triacryloyl-hexahydro-*s*-triazine and ethylene glycol bis(succinimidylsuccinate).

Keywords: Congo red, native α-proteins, dye binding, oligomerization

Congo red (CR) is a symmetrical sulfonated azo dye with a hydrophobic center consisting of a biphenyl group spaced between the negatively charged sulfate groups. Staining with CR is a standard method used to examine *ex vivo* tissue sections for amyloid deposits (Westermark *et al.*, 1999). There are a number of diseases in which normally soluble proteins associate into regular insoluble amyloid fibrils. More than 20 different proteins have been identified in disease-associated amyloid deposits (Buxbaum, 2003; Wu *et al.*, 2003). CR is also used for the characterization of amyloid fibrils *in vitro* (Ramirez-Alvarado *et al.*, 2000; Koga *et al.*, 2002; Kim et *al*, 2002; Cannon *et al.*, 2004; Ha & Park, 2005). Green birefringence under polarized light upon staining with CR together with fibrillar morphology and β-sheet secondary structure are the criteria that define a protein aggregate as an amyloid fibril (Nilsson, 2004). There is also a spectrophotometric assay for studying the interaction of CR with amyloid proteins (Klunk *et al.*, 1989; 1999). However, the mechanism of interaction between CR and amyloid fibrils is not well understood. It has been suggested to be a steric intercalation of CR molecules between β-sheets of amyloid fibrils or a combination of hydrogen bonding, hydrophobic and electrostatic interactions between the dye molecule and the fibril (Kim *et al.*, 2003; Nilsson, 2004).

Although CR is considered to have specific affinity for crossed β-structures present in amyloid fibrils or β-sheets in native proteins (Westermark *et al.*, 1999; Piekarska *et al.*, 2001; Krol *et al.*, 2005), it has been shown that CR can also bind to native α-proteins such as citrate synthase and interleukin-2. In addition, it has been demonstrated that CR binding does not induce changes in secondary structure, e.g. from α to β of those proteins (Khurana *et al.*, 2001).

In this paper we report data on the interaction between CR and native α-proteins-recombinant human growth hormone (hGH) and recombinant human interferon-α2b (IFN-α2b).

MATERIALS AND METHODS

Materials. hGH and IFN-α2b were provided by Biotechna company (Lithuania). CR was obtained from Reachim and recrystallized from 50% ethanol. Protein markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) were obtained from Pharmacia. 1,3,5-Triacryloyl-hexahydro-*s*-triazine (TAT) was prepared as described by Gresham and Steadman (1949) and recrystallized from water. Ethylene glycol bis(succinimidylsuccinate) (EGS) was obtained from Sigma.

⋆Presented at the 18th Polish Peptide Symposium, 4–8 September, 2005, Wrocław, Poland.

Abbreviations: CR, Congo red; cv, coefficient of variations; EGS, ethylene glycol bis(succinimidylsuccinate); hGH, recombinant human growth hormone; IFN-α2b, recombinant human interferon-α2b; TAT, 1,3,5-triacryloyl-hexahydro-*s*-triazine.

Absorption difference spectroscopy. Difference spectra were recorded on an Ultrospec 4000 spectrophotometer (Pharmacia Biotech) provided with SWIFT II software in the wavelength region 400–800 nm and using a path length of 1 cm. Difference spectral titrations were performed in 0.025 M phosphate buffer, pH 6.9 or pH 8.0, and 0.05 M borate buffer, pH 9.2, at 25°C. The sample cuvette contained 6.4 μM hGH, while the dye concentration ranged from 5 to 90 μM. The dye concentration in the reference cuvette was the same as that used for the complex formation.

Difference spectra for CR binding to IFN-α2b were recorded in 0.025 M phosphate buffer, pH 6.9: 1. at a constant IFN-α2b concentration of 19.8 μM, while the dye concentration ranged from 10 to 70 μM; 2. at the molar ratio 1:1 of [IFN-α2b]/[CR]. The concentrations were 9.9, 19.8 and 29.7 μM.

Isolation of hGH–CR complex by gel-permeation chromatography on Sephadex G-50. Solution (6.5 ml) containing 39.2 μM hGH and 170 μM CR in 0.05 M borate buffer, pH 9.2, was applied to a Sephadex G-50 column (1.5×19 cm), previously equilibrated with the same buffer. The column was eluted at 4°C using the equilibrium buffer at a flow rate of 0.8 ml/min. The eluate was monitored at 280 nm and 490 nm.

Chemical cross-linking by TAT. A total of 0.5 ml of a reaction mixture containing 14 μM hGH, 60 μM or 120 μM CR and 1.76 mM TAT in 0.05 M borate buffer, pH 9.2, was incubated for 3 h at 30°C. Subsequently, the reaction mixture was analyzed by SDS/PAGE for identification of cross-linked protein forms.

Chemical cross-linking by EGS. A total of 0.5 ml of a reaction mixture containing 14 μM hGH, 138 μM CR and 14.7 mM EGS (previously dissolved in dimethyl sulfoxide) in 0.025 M phosphate buffer, pH 7.4, was incubated for 2.5 h at 30°C and analyzed as above.

SDS/PAGE was carried out under reducing conditions according to the method of Laemmli (1970). The acrylamide concentration was 15%. Proteins were stained with Coomassie Brilliant Blue R-250. The gels were scanned and the ratio of hGH forms was estimated using the UN-SCAN-IT software.

RESULTS AND DISCUSSION

Absorption difference spectroscopy in combination with gel-permeation chromatography and chemical cross-linking techniques were used to study the interaction of CR (Fig. 1) with native α-proteins.

The visible absorption spectrum of CR has a maximum at 490 nm (Fig. 2, curve 1). Before recording of difference spectra the linearity of the absorption *vs.* concentration plot (obedience of the Beer-Lambert law) was checked for dye concentration up to 100 μM in both Na-phosphate buffer (pH 6.9 and 8.0) and borate buffer, pH 9.2. No spectral perturbations were revealed. The molar absorption coefficient at 525 nm was calculated from the calibration graph of Congo red absorbance *vs.* dye concentration and found to be 24000 M^{-1} cm^{-1} . The visible difference spectrum indicated that a shift of the maximum by about 35 nm towards longer wavelengths occurs when hGH is added (Fig. 2, curve 2). These spectral changes suggest that CR forms a complex with hGH. For characterization of the dye-binding process 6.4 μM hGH was titrated with an increasing amount of CR at pH 6.9, 8.0 and 9.2. The type of spectra obtained when the absorbance of the mixtures of dye and hGH was measured against a dye solution of the same concentration can be seen in Fig. 3. The intensity of the 525 nm peak at each difference spectrum was plotted *vs.* the dye concentration (Figs. 4–6). The equation described previously was used to calculate the parameters of the complex of hGH–CR (Bumeliene *et al.*, 2003):

$$\Delta A = \frac{1}{2}(E_{PL} - E_L)(L_0 + nP_0 + K_d - \sqrt{(L_0 - nP_0)^2 + K_d 2(L_0 + nP_0) + K_d{}^2}),$$

where K_d is the dissociation constant of the hGH–CR complex; L_0 and P_0 are initial concentrations of CR and hGH, respectively; n is the number of dye-binding sites per protein molecule; E_L and E_{PL} are molar absorption coefficients of CR and the hGH–CR complex; respectively; ΔA is the difference absorbance at 525 nm.

The experimental data (Figs. 4–6) were fitted to an approximating equation given above using nonlinear least-squares method implemented in the Mathcad 5.0 program package. The values of K_d and E_{PL} with the lowest coefficient of variation (cv) were found for n to be equal to 1. K_d and E_{PL} calculated by this method are presented in Table 1. K_d shows a tendency to decrease while pH is increasing. It is known that pI of hGH is approx. 5.3 (Pearlman & Bewley, 1993). The negative charge of the protein molecule increases with pH increasing above the value of pI. Keeping in mind that sulfonic acid groups are also negatively charged at high pH it could be supposed that electrostatic interaction between both dye and protein molecules is not prevalent.

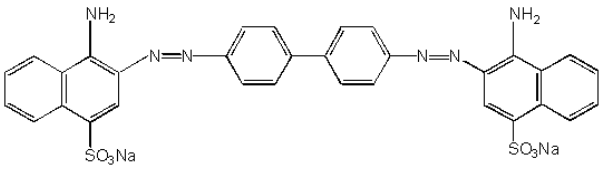

Figure 1. Chemical formula of Congo red.

Table 1. The dissociation constants (K_d) and molar absorption coefficients (E_{PL}) of hGH–CR complex at different pH values, 25°C

pH	K_d, μM	E_{PL}, $M^{-1}cm^{-1}$; λ=525 nm	cv, %
6.9	21.9	62000	8.4
8.0	15.7	57000	10.9
9.2	7.9	56000	10.9

In a previous paper (Sereikaite & Bumelis, 2006) successful isolation of both the human growth hormone and interferon-α2b complexes with the archetypal textile dye Cibacron Blue F3G-A, which is widely used as a ligand for chromatographic purposes, was shown. To confirm the formation of the hGH–CR complex we attempted to isolate it by gel-permeation chromatography on Sephadex G-50. The visible absorption spectrum of the isolated complex showed a shift to the long-wave side in comparison to free dye (Fig. 7).

Moreover, CR not only binds to the α-protein hGH, but also causes the generation of hGH dimers in solution (Fig. 8). It is well-known that formation of protein oligomers in solution can be investigated by cross-linking of the subunits and subsequent SDS-electrophoresis (Loster & Josic, 1997). The ability of CR to induce dimerization of hGH was tested using TAT, a trifunctional chemical cross-linking agent previously demonstrated to be extremely stable, simple and convenient to cross-link oligomeric species of protein (Dienys *et al.*, 1998). We have previously described its use for defining the oligomeric state of such well-characterized monomeric, dimeric, or trimeric proteins, as human growth hormone, interferon-γ, and tumor necrosis factor α, respectively (Dienys *et al.*, 1998; Kvederas *et al.*, 2000). Here we found that the dimer yield of hGH in the presence of CR was slightly dependent on dye concentration. The percentage of the dimeric form of total protein was 13% and 17% in the molar ratio 1:4 and 1:8 of hGH/CR, respectively. CR runs as a red band approximately as a 30-kDa protein. It is known that CR can form ribbon-like micelles (Piekarska *et al.*, 1999; Stopa *et al.*, 2003). Apparently, self-association of the dye takes place in the presence of sodium dodecyl sulfate. The cross-linking reaction results were also reproduced using the well-known cross-linker EGS at neutral pH 7.4. The phenomenon of dye-induced self-oligomerization of proteins was reported previously. The ability of CR to induce oligomerization of native proteins such as pectate lyase, carbonic anhydrase, β-lactoglobulin, interleukin-2 and lysozyme was found by Khurana and co-workers

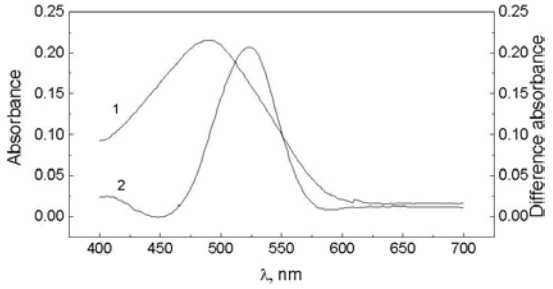

Figure 2. Absorption spectra of CR
(1) CR of 5 μM in 0.05 M borate buffer, pH 9.2 (2) Typical difference spectrum for CR binding to hGH. Both the sample and the reference cuvettes contained 50 μM CR in 0.05 M borate buffer, pH 9.2, and the sample cuvette also contained 6.4 μM hGH.

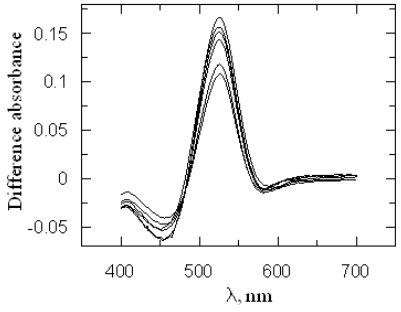

Figure 3. Typical difference spectra of CR dye binding to hGH in 0.025 M phosphate buffer, pH 8.0.
The sample cuvette contained 6.4 μM hGH whereas the dye concentration in both sample and reference cuvettes ranged from 15 to 70 μM.

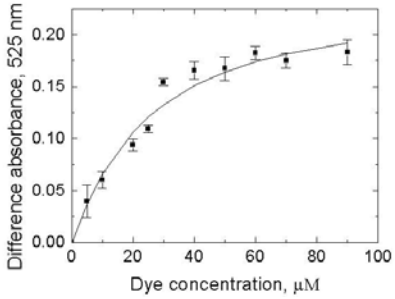

Figure 4. Titration at λ = 525 nm of 6.4 μM hGH with CR in 0.025 M phosphate buffer, pH 6.9, 25°C (▪).
Experimental data which are presented with standard deviations calculated from results of three parallel experiments; (–) the theoretically calculated curve for one dye-binding site per protein molecule.

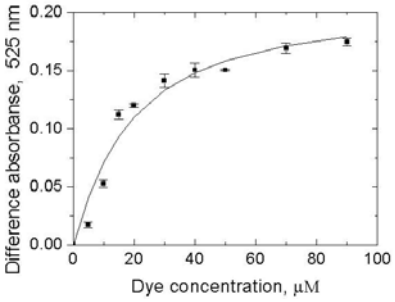

Figure 5. Titration at λ = 525 nm of 6.4 μM hGH with CR in 0.025 M phosphate buffer, pH 8.0, 25°C (▪).
Experimental data which are presented with standard deviations calculated from results of three parallel experiments; (–) the theoretically calculated curve for one dye-binding site per protein molecule.

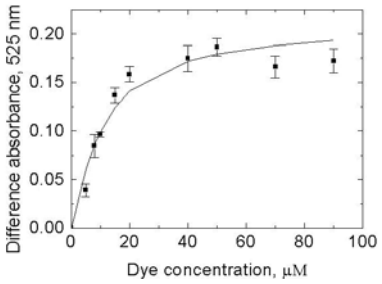

Figure 6. Titration at λ = 525 nm of 6.4 μM hGH with CR in 0.05 M borate buffer, pH 9.0, 25°C (▪).
Experimental data which are presented with standard deviations calculated from results of three parallel experiments; (–) the theoretically calculated curve for one dye-binding site per protein molecule.

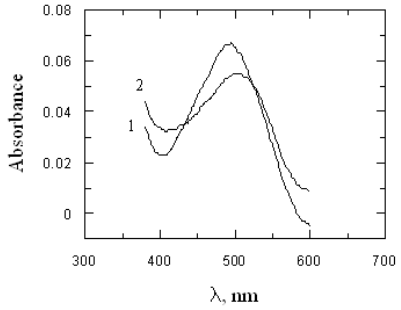

Figure 7. Visible absorption spectra of hGH–CR complex and CR
(1) Free CR in 0.05 M borate buffer, pH 9.2. (2) Complex of hGH–CR in 0.05 M borate buffer, pH 9.2.

(2001). In addition, it was revealed that other well-known dyes also induce protein oligomerization. Cibacron blue F3G-A promotes the generation of INF-α2b dimers at pH 5.0, while in water solutions at that pH this protein is usually monomeric (Sereikaite & Bumelis, 2006), α-synuclein is oligomerized by Coomassie Brilliant Blue (Lee *et al.*, 2001) and eosin (Shin *et al.*, 2000). In contrast, CR and Cibacron Blue F3G-A did not cause the oligomerization of α-synuclein or hGH, respectively. It could be considered that the protein-dye interaction is a specific process depending on both the protein and the dye. In all cases mentioned above cross-linking was used as a reliable tool for probing the oligomeric structure of the protein in the presence of the dye.

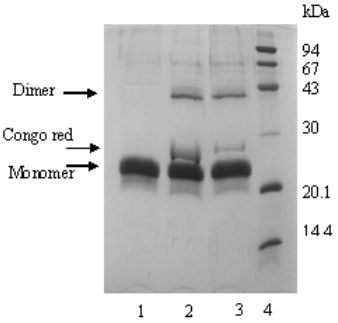

Figure 8. SDS/PAGE (15%) of hGH cross-linked by TAT in the presence of CR.
Cross-linking conditions: 0.05 M borate buffer, pH 9.2, 30°C, 3h, initial concentrations: hGH, 14 μM; TAT, 1.76 mM. Lane 1, hGH control sample in the absence of CR; lanes 2 and 3, molar ratio hGH/CR, 1:8 and 1:4, respectively; lane 4, protein markers.

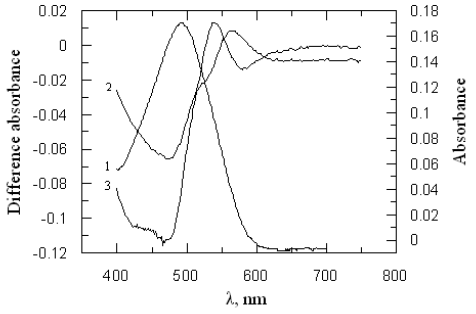

Figure 9. Absorption spectra of CR (1) CR of 4 μM in 0.025 M phosphate buffer, pH 6.9, and (2, 3) difference spectra of CR dye-binding to INF-α2b in 0.025 M phosphate buffer, pH 6.9.
The sample cuvette contained 19.8 μM INF-α2b whereas the dye concentration in both sample and reference cuvettes was 10 μM (2) or 70 μM (3).

CR was also found to form a complex with INF-α2b, but the dye interaction with this protein was more complicated. The difference spectra did not exhibit an isobestic point. The value of λ_{max} varied depending on the concentration ratio [dye]/[protein] (Fig. 9). In the case of equal of the dye and protein concentrations or in the presence of protein molar excess λ_{max} was at 560 nm. The maximum moved to the shorter wavelengths (to about 530–540 nm) in the excess of the dye. It is likely that more than one type of complex was formed.

Thus, CR probably lacks specificity for protein secondary structure. This conclusion is supported by the fact that binding of CR does not result in induction of β-structure (Khurana *et al.*, 2001). On the other hand, non-helical polypeptide portions could also be engaged in complex formation. In any case INF-α2b and hGH increase the number of α-proteins to which CR is able to bind. The CR binding to native α-proteins could be a reason of false-positive results using this dye as a diagnostic test for *in vitro* detection of fibrils (Khurana *et al.*, 2001).

REFERENCES

Bumeliene Zh, Sereikaite J, Bumelis V-A, Smirnovas V, Gedminiene G, Braziunaite L, Bajorunaite E (2003) Determination of the dissociation constant and stoichiometry of a complex of the protein interferon α-2b with Cibacron Blue F3G-A. *J Anal Chem* **58**: 1038–1041.

Buxbaum JN (2003) Diseases of protein conformation: what do *in vitro* experiments tell us about *in vivo* diseases. *Trends Biochem Sci* **28**: 585–592.

Cannon MJ, Williams AD, Wetzel R, Myszka DG (2004) Kinetic analysis of beta-amyloid fibril elongation. *Anal Biochem* **328**: 67–75.

Dienys G, Sereikaite J, Gavenas G, Kvederas R, Bumelis VA (1998) Cross-linking of protein subunits by 1,3,5-triacryloyl-hexahydro-*s*-triazine. *Bioconjugate Chem* **9**: 744–748.

Gresham TL, Steadman TR (1949) Triazines from formaldehyde and nitriles. *J Am Chem Soc* **71**: 1872–1873.

Ha C, Park CB (2005) Template-directed self-assembly and growth of insulin amyloid fibrils. *Biotechnol Bioeng* **90**: 848–855.

Khurana R, Uversky VN, Nielsen L, Fink AL (2001) Is Congo red an amyloid-specific dye. *J Biol Chem* **276**: 22715–22721.

Kim YS, Randolph TW, Stevens FJ, Carpenter JF (2002) Kinetics and energetics of assembly, nucleation, and growth of aggregates and fibrils for an amyloidogenic protein. *J Biol Chem* **277**: 27240–27246.

Kim YS, Randolph TW, Manning MC, Stevens FJ, Carpenter JF (2003) Congo red populates partially unfolded states of an amyloidogenic protein to enhance aggregation and amyloid fibril formation. *J Biol Chem* **278**: 10842–10850.

Klunk WE, Pettegrew JW, Abraham DJ (1989) Two simple methods for quantifying low-affinity dye-substrate binding. *J Histochem Cytochem* **37**: 1293–1297.

Klunk WE, Jacob RF, Mason RP (1999) Quantifying amyloid β-peptide (Aβ) aggregation using the Congo red-Aβ (CR-Aβ) spectrophotometric assay. *Anal Biochem* **266**: 66–76.

Koga T, Taguchi K, Kogiso M, Kobuke Y, Kinoshita T, Higuchi M (2002) Amyloid formation of native folded protein induced by peptide-based graft copolymer. *FEBS Lett* **531**: 137–140.

Krol M, Roterman I, Piekarska B, Konieczny L, Rybarska J, Stopa B, Spolnik P, Szneler E (2005) An approach to understand the complexation of supramolecular dye Congo red with immunoglobulin L chain lambda. *Biopolymers* **77**: 155–162.

Kvederas R, Mistiniene E, Sereikaite J, Luksa V, Dienys G, Bumelis VA (2000) Association of tumor necrosis factor alpha in water solution. *Biologija* **2**: 253–256.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Lee D, Lee EK, Lee JH, Chang CS, Paik SR (2001) Self-oligomerization and protein aggregation of α-synuclein in the presence of Coomassie Brilliant Blue. *Eur J Biochem* **268**: 295–301.

Loster K, Josic D (1997) Analysis of protein aggregates by combination of cross-linking reactions and chromatographic separations. *J Chromatogr B* **699**: 439–461.

Nilsson M (2004) Techniques to study amyloid fibril formation *in vitro*. *Methods* **34**: 151–160.

Pearlman R, Bewley TA (1993) Stability and characterization of human growth hormone. In *Stability and Characterization of Protein and Peptide Drugs: Case Histories* (Wang YJ, Pearlman R eds) pp 1–58, Plenum Press, New York.

Piekarska B, Rybarska J, Stopa B, Zemanek G, Krol M, Roterman I, Konieczny L (1999) Supramolecularity creates nonstandard protein ligand. *Acta Biochim Polon* **46**: 841–851.

Piekarska B, Konieczny L, Rybarska J, Stopa B, Zemanek G, Szneler E, Krol M, Nowak M, Roteman I (2001) Heat-induced formation of a specific binding site for self-assembled Congo red in V domain of immunoglobulin L chain lambda. *Biopolymers* **59**: 446–456.

Ramirez-Alvarado M, Merkel JS, Regan L (2000) A systematic exploration of the influence of the protein stability on amyloid fibril formation *in vitro*. *Proc Natl Acad Sci* **97**: 8979–8984.

Sereikaite J, Bumelis VA (2006) Examination of dye-protein interaction by gel-permeation chromatography. *Biomed Chromatogr* **20**: 195–199.

Shin HJ, Lee EK, Lee JH, Lee D, Chang CS, Kim YS, Paik SR (2000) Eosin interaction of α-synuclein leading to protein self-oligomerization. *Biochim Biophys Acta* **1481**: 139–146.

Stopa B, Piekarska B, Konieczny L, Rybarska J, Spolnik P, Zemanek G, Roterman I, Krol M (2003) The structure and protein binding of amyloid-specific dye reagents. *Acta Biochim Polon* **50**: 1213–1227.

Westermark GT, Johnson KH, Westermark P (1999) Staining methods for identification of amyloid in tissue. *Methods Enzymol* **309**: 3–25.

Wu DW, Lou JY, Chen J, Fei L, Liu GJ, Shi XY, Lin HT (2003) A case report of localized gastric amyloidosis. *World J Gastroenterology* **9**: 2632–2634.