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Spectroscopic study of pH and solvent effects on the structure of Congo red and its binding mechanism to amyloid-like proteins

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Abstract–The pH and solvent effects on the structure of the azo dye Congo red were investigated. The absorption, resonance Raman and NMR spectra give evidence of the existence of different protonated dye molecules in acidic solutions. There exist a red form with an azoic structure and at least three blue forms with quinoid structures. The position of the equilibrium between the azoic and quinoid structures depends on the solvent and the pH and is related to the capability of the solvent to form three and two dimensional solvation structures, respectively.

Spectral variations similar to those observed for Congo red dissolved in organic solvents accompany binding of the dye to amyloid-like proteins. This observation leads to the suggestion that the dye molecules must experience similar changes in environment in both cases.

INTRODUCTION

THE azo dye Congo red (Fig.1) is used in histochemical analyses of human tissue for the diagnosis of amyloid protein deposits characteristic of a wide variety of diseases including Alzheimer's disease [1]. The amyloid fibres have a high affinity for the Congo red molecule and exhibit a yellow/green birefringence and a characteristic dichroism when stained with this dye [2]. Several investigations have been carried out by histochemical [3-5] and spectroscopic [6-10] methods to define the nature of this specific staining. However, the molecular mechanism of the interaction between Congo red and the amyloid proteins is still not entirely understood.

Previous published studies barely took into account the chemical properties and the different possible structures of the Congo red molecule. However, in the course of investigations carried out in this laboratory of the binding mechanism of Congo red to amyloid-like proteins, e.g. poly-L-lysine and insulin [8-10], it was found that in order to understand the state of the dye molecule adsorbed on the protein it is necessary to have a comprehensive knowledge of the behaviour of the free molecule. In this paper we present the results of our studies of the pH and solvent effects on the structure of the Congo red molecule and their relation to the effects in the bound state. According to REEVES [11,12] there are similarities between the spectral changes that accompany binding of dye molecules to substrates and those which occur when the molecules are placed in organic solvents. Therefore, it was felt that the study of the behaviour of the Congo red molecule in organic solvents could help to understand its interactions with proteins.

Congo red is an acid-base indicator which shows a colour transition from red to blue below a pH of 5. This is generally understood to be a consequence of a change in the molecular structure upon protonation accompanied by the formation of resonance structures [13,14]. So far these structural changes have been studied mostly by measuring the electronic absorption spectra as a function of the pH and the solvent [13,15,16]. In the present work we report resonance Raman and ^{1}H and ^{13}C NMR spectra which give clear evidence of the different species present in neutral and acidic Congo red solutions.

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EXPERIMENTAL

Congo red was obtained as its disodium salt from Aldrich and used as supplied. The organic solvents were of spectrophotometric grade. The UV/vis and resonance Raman measurements were carried out with 5×10^{-6} M Congo red solutions in a 0.1 M citric acid buffer. For the solutions in organic solvents, 75 % of the water content of the buffer was replaced by the solvent. The NMR measurements were made with saturated solutions of the Congo red salt and the Congo red acid, respectively, in DMSO.

The absorption spectra were recorded using a Cary 1 Varian spectrophotometer. The resonance Raman spectra were obtained with a Dilor RT triple-monochromator spectrometer using Ar^+ (514.5 nm) and Kr^+ (647.1 nm) exciting lines. The NMR spectra were measured on a 400 MHz Bruker NMR spectrometer with a resolution of 0.6 Hz/pt and at a temprature of 294 K.

RESULTS AND DISCUSSION

pH effects in aqueous solution

In a 5×10^{-6} M aqueous solution in a citric acid buffer at a neutral pH, Congo red appears orange red and its absorption spectrum exhibits bands at 237 nm, 341 nm and 489 nm. The band at 489 nm, which is assigned to a π-π^* transition of the azo group [17], is the one most affected by a decrease in pH. The spectra in Fig. 2A show the evolution of this band as a function of the pH. At a pH of 5.3 the solution becomes violet and the spectrum shows a band at 520 nm and a broad band system with maxima at 665 nm and 730 nm. With a further increase in acidity the intensity of the band at 520 nm decreases whereas the other two maxima show a slight increase in intensity. At a pH below 2.7, Congo red appears dark blue and has an increasing tendency to precipitate out of solution.

The colour transition of Congo red at a pH of 5.3 is caused by a protonation of the molecule resulting in the formation of resonance structures (see Fig.1).

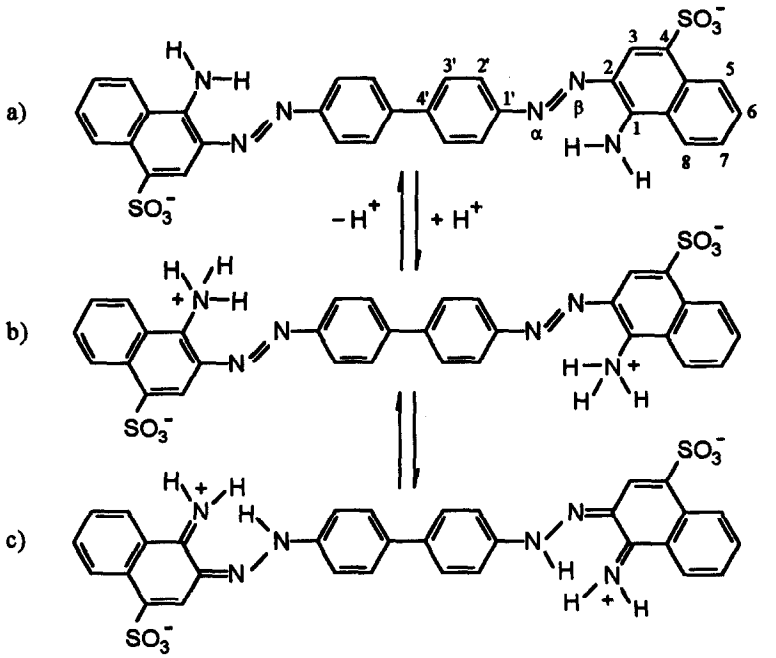

Fig. 1. Protonation and tautomeric equilibrium of the Congo red molecule in acidic solution

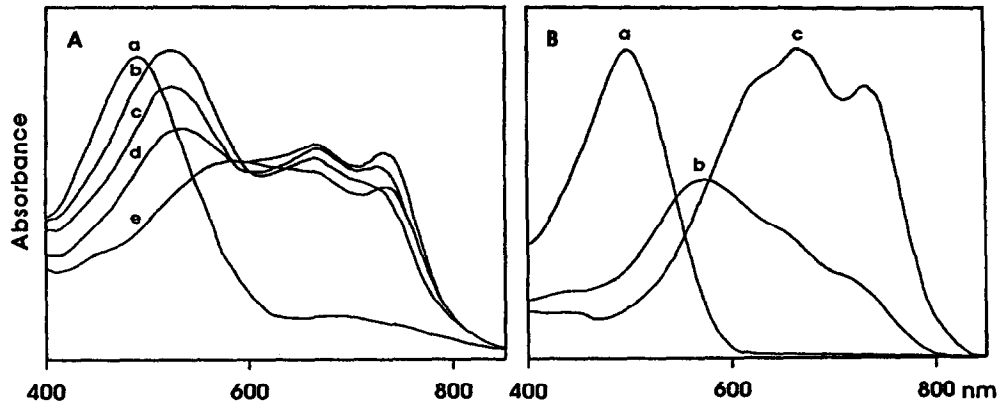

Fig. 2. Absorption spectra of Congo red in aqueous buffer solution, (A) at various pH values: (a) 5.7, (b) 5.3, (c) 4.5, (d) 3.5, (e) 2.0; (B) solution brought quickly to a pH of 3.0: (a) after heating to 90 °C, (b) just after acidification, (c) after slow cooling of the heated solution

There are two tautomers of the protonated Congo red molecule, an ammonium form with the proton attached to the amino nitrogen (b) and an azonium form, where the proton is added to the α-azo nitrogen (c). In the acidic solution both forms are present in a tautomeric equilibrium mixture.

We assign the absorption band at 520 nm to the ammonium form. In this form a conjugation of the azo linkage with the aromatic system is hindered and the azoic structure is preserved. Consequently the absorption spectrum of this tautomer should be closly similar to that of the Congo red molecule in neutral and basic solution [18]. The band system between 600 nm and 800 nm is assigned to the azonium form, where the formation of a quinoid structure causes an absorption at longer wavelengths.

The tautomeric equilibrium between the ammonium and the azonium form is stabilized by the formation of a six membered chelate ring with the amino group and the azo group and the oscillation of a proton between them. However, the installation of this equilibrium at a pH of 5.3 seems to be kinetically hindered because the initial absorption spectrum which is similar to the one at a pH of 5.7 changes over a period of about 10 hours to give the final spectrum (Fig. 2A(b)). After that time the solution is stable for more than two days. Further stepwise acidification leads to the changes shown in Fig. 2A. On the other hand, when a Congo red solution is brought very quickly to a pH of 3.0, a purple solution is obtained with an absorption spectrum shown in Fig. 2B(b). This spectrum has an absorption maximum at 570 nm and is similar to those reported hitherto in the literature [8,15,16]. We suppose that this quickly acidified solution is not in equilibrium due to the formation of aggregates by the protonated dye molecules. This aggregation is manifested in the decrease in intensity of the absorption band. When the solution was heated to 90 °C, it became orange red again due to a resolution of the aggregates and a shift of the equilibrium in the direction of the deprotonated molecule. Its absorption spectrum (Fig. 2B(a)) is the same as that of Congo red in a neutral solution. After slow cooling, the colour of the solution turned to azure blue and the absorption spectrum (Fig. 2B(c)) exhibited only the band system assigned to the azonium form with an additional shoulder at 620 nm. The existence of three maxima implies that there are probably several different protonated molecules with quinoid structures. This coexistence has also been suggested by other authors [15,19] and will be demonstrated below.

Resonance Raman spectra confirm the presence of azoic and quinoid structures in acidic Congo red solutions. The assignments of the bands are given in Table 1

Table 1. Frequencies (cm^{-1}) and assignments of the resonance Raman spectra of Congo red solutions, (a) pH= 5.3, excitation 514.5 nm, (b) pH= 5.3, excitation 647.1 nm, (c) pH= 3.0, excitation 514.5 nm

a)	b)	c)	Assignments
1157	1157		ν C-N=
	1179	1182	ν N-N
	1266	1267	
	1292	1289	ν C-N-
		1306	
		1322	
1376	1375	1374	ν N=N
1401	1400	1400	
1453	1457	1458	ν C-C aromatic ring
	1566	1567	ν C=N-
1592	1592	1592	ν C-C aromatic ring

referring to previous works on vibrational spectra of azo dyes [20-22]. The spectrum of the solution at a pH of 5.3 obtained at excitation wavelengths of 514.5 nm (a), which is in resonance with the absorption at 520 nm, showed three dominant bands at 1157, 1376 and 1401 cm^{-1} which are assigned to C-N= and N=N stretching vibrations of an azoic structure. The other bands at 1453 and 1592 cm^{-1} have been assigned to aromatic ring modes. The spectrum obtained with an excitation wavelength of 647.1 nm (b) showed, in addition to the bands of the azoic structure, other bands at 1179, 1266, 1292 and 1566 cm^{-1} which are assigned to a quinoid structure. In the spectrum of the azure blue solution (c) these bands were the most intense and the N=N stretching band of the azoic structure has almost disappeared. Hence, one can conclude that quinoid structures dominate in the azure blue solution. However a distinction between different protonated molecules cannot be made from the resonance Raman spectra.

solvent effects

In organic solvents one observes two major spectral changes for Congo red. Firstly, there is a bathochromic shift of the azo band in the absorption spectrum. Secondly, the colour transition of the Congo red solution occurs at a much lower pH than in an aqueous solution.* This latter effect is accompanied by an elimination or compression of the colour transition interval.

Table 2 gives the absorption maxima of Congo red in different solvents. The bathochromic shift of the azo band has several causes. Besides the refractive index of the solvent, this red shift is influenced by the intramolecular hydrogen bond between the azo and the ortho-amino group. The change from an aqueous environment to a hydrophobic one, leads to an enhancement of this hydrogen bond and causes an absorption at lower frequencies. Conversely, the dye methyl orange where the amino group is in para-position shows a blue shift of the azo band in organic solvents [11].

The absorption spectra of Congo red in the organic solvents at a pH of 2.0 still show only the band characteristic of the azoic structure. With further acidification the colour of the solutions eventually turns to blue. This transition occurs at a pH and with a rate dependent on the solvent. For methanol, ethanol and acetonitrile the colour change

* Although the pH measurements of solutions containing non-aqueous solvents may not be strictly comparable we can consider them adequate for estimations of the trend.

Table 2. Absorption maxima of Congo red in different solvents

Solvent	$^{20}n_D$	Wavelengths in nm		
water	1.3333	237	341	489
methanol	1.3286	239	339	503
ethanol	1.3616	237	338	507
acetonitrile	1.3441	235	339	509
acetone	1.3591	+	+	515
DMSO	1.4783	+	344	532

+ perturbations due to solvent absorptions

happens within a few minutes at a pH of about 1.5. Fig. 3 shows the repetitive scan absorption spectra of Congo red in ethanol (A) at a pH of 1.5. There is a continuous decrease in intensity of the azo band and an increase of the band system assigned to the quinoid structures. Eventually, only the bands of the blue form exist.
The existence of an almost clear isobestic point is indicative of a single-step transition as shown in Fig.1. But one can deduce from the absorption spectra in acetone (B) and in DMSO (C) that there must be other equilibria involved. The repetitive scan spectra of a Congo red solution in acetone at a pH of 1.0 show at first, that of the three bands of the quinoid forms, the band at 620 nm dominates. This band must originate from a protonated form of the Congo red molecule, which is more stabilized by the solvent than are other forms. As can be seen in Fig. 3C, in DMSO only this structure exists and it is stable even at the very low pH of 0.5. Unfortunately, this Congo red solution exhibits a strong fluorescence, so it was not possible to take a resonance Raman spectrum. But considering the low pH and the high solvation power of acetone and DMSO for cations, this structure must be a highly protonated one.

The delay of the colour transition in the organic solvents is due to a weaker stabilization of the quinoid structure of the protonated Congo red molecule than in water. This interpretation is similar to the findings for dyes which show an azo-hydrazone tautomerism [23,24]. According to REEVES [11,25] this effect can be explained by the capability of water to form three dimensional hydrogen bonding regions. The hydrophobic parts of the dye molecule dissolved in water enhance these hydrogen bonded structures in their immediate vicinity and cause the water molecules to form an ice-like structure. In these water cavities the quinoid form can assume a more favourable conformation and is stabilized. Solvents which are either two dimensional (ethanol) or unstructured (aprotic and unpolar) accumulate with their nonpolar moieties in direction of the hydrophobic regions of the dye molecule. The result is a diffuse interface between dye and bulk solvent with low polarity. Under these conditions the azo form is more stabilized.

The results from the absorption spectra were affirmed by the ^1H and ^{13}C NMR spectra of the Congo red disodium salt and the blue Congo red acid dissolved in the unstructured aprotic solvent DMSO. The ^1H NMR spectra are shown in Fig. 4 and the chemical shifts and the assignments are given in Table 3. The assignments were made by decoupling experiments and referring to literature data for azo dyes and naphtalene derivatives [25-27]. The ^1H NMR spectrum of the Congo red salt (Fig. 4(a)) shows a signal at 7.73 ppm which is assigned to the amino group. This signal disappears in the spectrum of the Congo red acid (Fig. 4(b)), where one observes a triplet with a low intensity and a coupling constant $^1J(^{14}NH)$ of 51.3 Hz at 7.15 ppm. This feature is a typical pattern of a protonated amino group [28]. The other chemical shifts are identical for the salt and the acid, indicating that the aromatic bond systems are

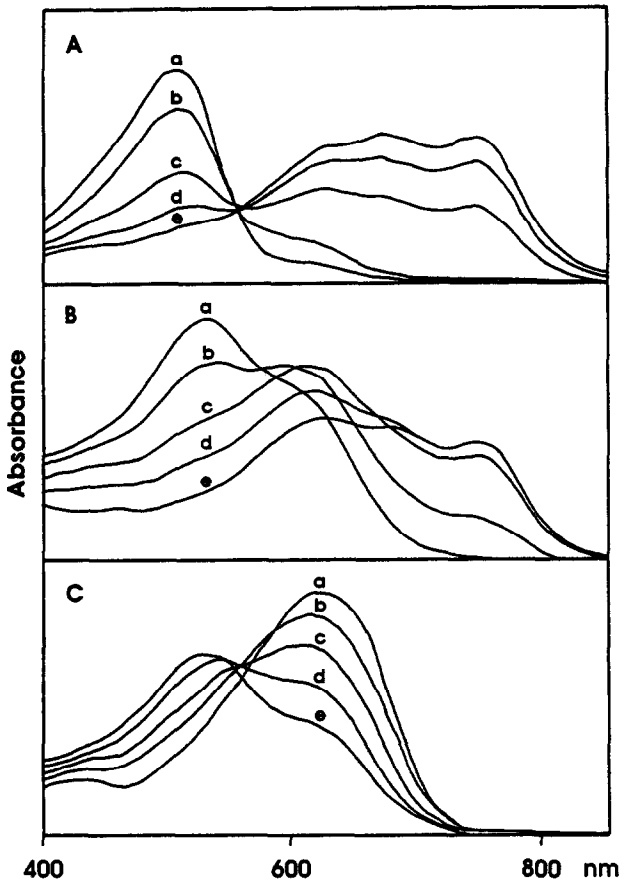

Fig. 3. Absorption spectra of Congo red in various solvents as a function of time and pH, (A) ethanol pH=1.5: (a) 0 min, (b) 1 min, (c) 4 min, (d) 5 min and (e) 8 min; (B) acetone pH=1.0: (a) 1 min, (b) 10 min, (c) 30 min, (d) 60 min and (e) 110 min; (C) DMSO pH:(a) 0.5, (b) 0.7, (c) 0.9, (d) 1.2 and (e) 1.4

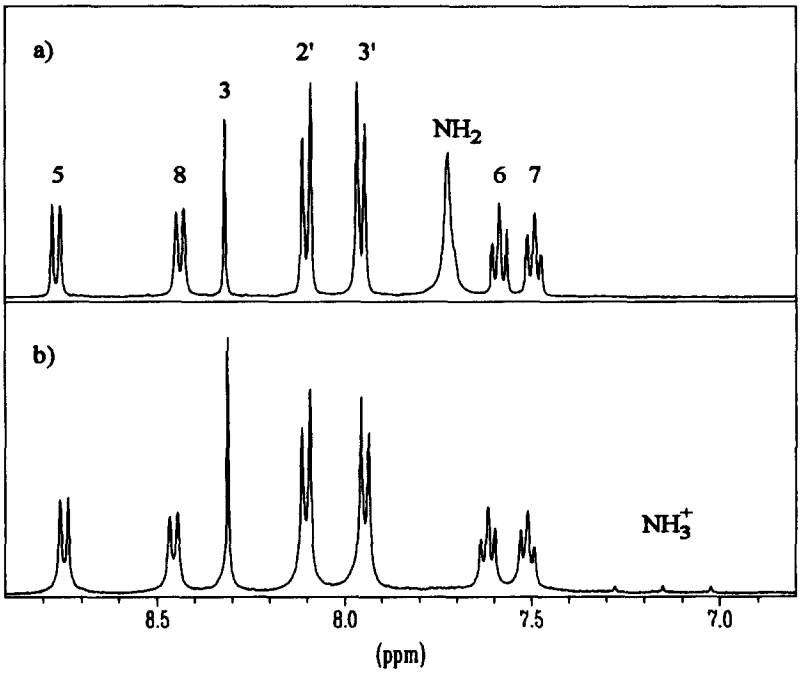

Fig. 4. ^{1}H-NMR spectra of Congo red (a) and the blue Congo red acid (b) in DMSO-d_6

unaltered and that the Congo red acid exists in DMSO mostly in the azoic structure protonated at the amino group.

The ^{13}C NMR spectra give a similar result. The greatest difference in the ^{13}C chemical shift values for the salt and the acid is observed for the signal of the C(1) atom bound to the amino group. It shows a down field shift of 1.65 ppm for the acid which is due to the deshielding effect by the positive charged ammonium group.

Table 3. ^{1}H and ^{13}C chemical shifts (ppm) of Congo red salt and Congo red acid in DMSO-d_6

H/C number	Congo red salt		Congo red acid	
	δ (^{1}H)	δ (^{13}C)	δ (^{1}H)	δ (^{13}C)
1		145.85		147.50
2		132.53		133.19
3	8.32	117.34	8.32	116.95
4		131.77		132.00
5	8.77	128.22	8.75	128.80
6	7.59	124.79	7.62	125.26
7	7.49	128.03	7.51	128.37
8	8.44	123.68	8.46	124.09
4a		128.96		129.00
8a		124.14		124.14
1'		152.21		151.07
2'	8.11	122.81	8.11	122.47
3'	7.96	127.38	7.95	127.45
4'		139.89		139.71
NH_2	7.73			
NH_3^+			7.15*	

* $^{1}J(^{14}NH)$= 51.3 Hz

Conclusions

This study has shown that the equilibrium between the different resonance structures of the protonated Congo red molecule depends strongly on the properties of the solvent. The spectral changes which occur going from aqueous solutions to organic solvents can be explained by hydrophobic interactions between the dye molecules and the solvent.

In previous studies we observed similar spectral changes for the binding of Congo red to amyloid-like proteins (poly-L-lysine, insulin) [8-10]. The absorption spectrum of the bound Congo red molecule showed a large bathochromic shift of the azo band and the molecule was stabilized in its azo structure over a wide pH range. These correlations show that hydrophobic interactions play an important role in the binding mechanism of the Congo red molecule to amyloid and amyloid-like proteins. This importance of hydrophobic binding effects for the Congo red molecule was also demonstrated by Turnell and Finch [29]. Another major contribution to the binding should be van der Waals attractions due to the extensive aromatic system of the molecule [30]. Starting from these two fundamental binding mechanisms, hydrophobic interactions and van der Waals attractions, we can further investigate the factors which determine the very selective binding of Congo red to amyloid.

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