The Interaction of Acridine Orange with Poly(S-carboxymethyl-L-cysteine)

Toyoko Imae and Shoichi Ikeda

Department of Chemistry, Faculty of Science, Nagoya University, Chikusa, Nagoya 464

(Received April 25, 1977)

The interaction of Acridine Orange with poly(S-carboxymethyl-L-cysteine) has been investigated by observing the solubility behavior, absorption spectra, and circular dichroism of the mixture. When the dye is added to aqueous solution of the polymer, the mixture is homogeneous at higher pH, but precipitation takes place at lower pH; the threshold pH ranges from 3.5 to 6, depending on the mixing ratio. For [P]/[D] 1 or less, the mixture is again homogeneous at very low pH between 4 and 2, when no salt is added. Various type of circular dichroism are induced for the homogeneous solutions, which are characteristic of the bound dye species on the polymer and the structure of dye–polymer complex. The binding of dye with the polymer is mainly caused by electrostatic effect, and the solubility of the mixture is interpreted by considering the ionization of polymer and the binding of dye to the polymer.

The visible absorption spectra of Acridine Orange in water are characterized by two absorption bands at 492 and 470 nm, which are ascribed to the monomer and dimer, respectively, of the dye.[1] The relative abundance of the two forms of the dye depends on its concentration. A structure of antiparallel stacked type has been proposed and accepted[2,3] for the dimeric dye molecule. In highly concentrated dye solutions, higher aggregates of dye are formed, the absorption band shifting down to around 450 nm.

When Acridine Orange is mixed with poly(S-carboxymethyl-L-cysteine), its absorption spectra change remarkably from those of free dye in the position and intensity of visible band.[4,5] The peak position indicates the state of aggregation of dye molecules in solution, while the hypochromism suggests the binding of dye to the polymer.

It has also been found that the circular dichroism (CD) is induced on Acridine Orange at its absorption bands in the presence of poly(S-carboxymethyl-L-cysteine).[4,5] The induced CD spectra observed at various [P]/[D] (mole of polymer residue/mole of added dye) mixing ratios and pH can be classified into fundamental types or their combinations. The fundamental types are characterized with reference to the bound species of dye molecules on the polymer and the conformation of the polypeptide chain. The latter is deduced from the CD spectra associated with the electronic transitions of peptide groups.

The CD spectrum of Type I was induced on aggregates of dimeric dyes bound to the polymer in the β-conformation.[4,6] Type II CD spectrum was induced on a dissymmetric array of dimeric dyes bound to the randomly coiled polymer at low ionization.[5,7] The CD of Type III was characteristic of low [P]/[D] ratios and was induced on another kind of array of dimeric dyes bound to the randomly coiled polymer at high ionization. Other types of CD, Types II′ and III′, originate from bound monomeric dyes and are considered to be counterparts of Types II and III, respectively.

The experiments so far reported[4,5] were performed on solutions of weakly acidic or higher pH, since the dye–polymer mixture precipitated at lower pH. It was found that the mixture was again homogeneous at very low pH region and for low [P]/[D]. The present work concerns with the interaction of Acridine Orange with poly(S-carboxymethyl-L-cysteine), as revealed in the solubility behavior and circular dichroism.

Experimental

Poly(S-carboxymethyl-L-cysteine) (code E602) was the same sample as that previously used.[8] Low-molecular-weight poly(S-carboxymethyl-L-cysteine) (code E515) was also used for comparison.[8] Acridine Orange was purified from commercial zinc salt and obtained as a hydrochloride salt.[1,4]

The stock solution of dye was added to aqueous solution of polymer. The pH was then adjusted by HCl or NaOH, followed by dilution to the desired dye concentration. For the preparation of solutions at a desired ionic strength, NaCl was added to the stock solution of polymer. The mixed solutions were kept overnight before measurements.

The pH was measured with a Hitachi-Horiba F5-X pH Meter. Absorption spectra were recorded with a 5 mm quartz cell on a Hitachi 323 Recording Spectrophotometer. The CD measurements were performed by using a 10 mm cell on a Jasco J-20 Circular Dichrometer, which was calibrated by use of aqueous solution of d-10-camphor sulfonic acid.[9] Measurements were carried out at room temperature.

The molar extinction coefficient, ε_D, and the molar ellipticity, $[\theta_D]$, are expressed on the basis of the total molar concentration of added dye.

Results

Solubility and Phase Separation of Dye–Polymer Mixture. Figure 1 shows the pH region for various [P]/[D] values, indicating where the mixed solution of Acridine Orange and poly(S-carboxymethyl-L-cysteine) is homogeneous and where precipitation takes place. A red precipitate is formed when the polymer concentration is high, and a fine gel separates when the polymer concentration is low.

The pH region where the mixture is soluble changes with the [P]/[D] ratio; as [P]/[D] decreases, the pH of phase separation becomes higher. The mixture is soluble above pH 5.8, when [P]/[D] is 0.9. At [P]/[D] lower than 0.9, the pH of phase separation is lowered. When solutions of different concentrations are compared at a given [P]/[D], it is found that the more concentrated mixture precipitates at slightly higher pH.

When [P]/[D] is 1 or less, homogeneous solutions appear at pH between 4 and 2. The upper and lower pH of homogeneous solutions change parallel to the pH

P/D	D (M)	SOLVENT	pH (1–7)
25	4.94×10^{-5}	WATER	ppt — 3.44 — solu.
4	4.94×10^{-5}	WATER	4.22
2.	4.94×10^{-5}	WATER	5.12
1	4.94×10^{-5}	WATER	2.35 — 3.99 — 5.32
0.9	4.94×10^{-5}	WATER	5.80
0.8	4.94×10^{-5}	WATER	1.81 — 3.91 — 5.30
0.6	4.94×10^{-5}	WATER	3.66 — 5.21
2	9.89×10^{-5}	WATER	5.65
1	19.77×10^{-5}	WATER	2.88 — 4.39 — 5.95
1	4.94×10^{-5}	2×10^{-3} M NaCl	5.39
1	4.94×10^{-5}	2×10^{-1} M NaCl	5.15

Fig. 1. Solubility of Acridine Orange–poly(S-carboxy-methyl-L-cysteine) mixture in water and in aqueous NaCl solutions. ——, Solutions; ----, precipitation.

TABLE 1. WAVELENGTH, SIGN, AND MAGNITUDE OF CD OF VARIOUS TYPES[a]

I	II	III	II'	III'
	560+ w	557− w	533− s	545+ s
			492−sh	500+sh
466+ s	472− s	470+ s	465+sh	473−sh
433− m	415+ w	422− w	427+ m	421− m
	382− w	373+ w		
	342+ w	343− w	320− m	326+ m
	293−sh	294+sh	294+sh	298−sh
269+ s	275− s	277+ s	273+ m	278− m
252− m	251+ m	253− m	259+ m	250− m
	221+ m	222− m	229+ m	224− m
			213− m	208+ m

a) Wavelength is given in nm. Magnitude is classified into strong (s), medium (m), weak (w), and shoulder (sh).

of phase separation around pH 5.5. In the solution of the lowest [P]/[D], 0.6, precipitation does not take place even at pH 1.80. Redissolution occurs for [P]/[D] 1, even when the dye concentration and consequently the polymer concentration are increased by four times. Addition of 0.2 M NaCl prevents the mixture of [P]/[D] 1 from becoming homogeneous in the pH region 4—2, and yields extremely fine gels in this region.

Dye–Polymer Mixture at Higher pH Region. In a higher pH region where no phase separation occurs, the dye molecules on the complex for high [P]/[D] generally form dimers or higher aggregates, while those for low [P]/[D] exist as monomers. The blue shift of the main visible absorption band down to 450 nm occurs for solutions of [P]/[D] higher than 1, suggesting the formation of highly aggregated dye. In the solutions of [P]/[D] 1 and 0.9, the dye molecule is in different states, depending on the pH. For [P]/[D] 0.8 and 0.6, the absorption band of monomeric dye is always dominant. The observed molar extinction coefficient of the main absorption band of dye in the visible region shows hypochromism, which implies extensive binding of dye to the polymer.

The induced CD spectra drastically vary with pH and [P]/[D]. Table 1 gives the wavelength of CD bands belonging to different types of CD. For [P]/[D] higher than 4, the CD of Type I is induced at acidic pH.[4] A strong positive band is located at 466 nm, and a weak negative one at 433 nm. The CD of Type II, which is observed for solutions of [P]/[D] 2 or 1 at pH 6.8 or 5.8, consists of paired bands with a strong negative band at 472 nm and a weak positive band at 415 nm, and of an additional broad positive band around 560 nm. The CD is also induced even at the neutral or alkaline pH when [P]/[D] is lower than 4. It is characterized by three bands, having opposite signs to those of Type II. This type of CD has been called Type III. The other type of CD, Type III', occurs for solutions of [P]/[D] 0.9—0.6 at acidic pH. A positive band is manifest at 545 nm, and a negative one at 421 nm. Some of the spectral characteristics of the dye–polymer mixtures have already been reported.[5] The pH and [P]/[D] values for the induction of CD of various types are given in Table 2.

Dye–Polymer Mixture at Very Low pH Region. The mixtures of [P]/[D] 1 or less are homogeneous at pH 4—2, when no salt is added. However, thier absorption spectra appreciably change from those of the solutions mentioned above. As illustrated in Figs. 2 and 3, the absorption spectra for [P]/[D] 1, 0.8, and 0.6 have two peaks at 492 and 470 nm, and their extinction coefficients differ slightly from those for the free dye at the same concentration.[1,4] This suggests that only a few dye molecules are bound to the polymer.

In this region, the CD behavior is even more complicated. As shown in Fig. 2, the CD for [P]/[D] 1 belongs to Type II at the most acidic pH. Upon raising pH, the three CD bands in the visible region change their signs, while the ultraviolet bands keep them unchanged. However, the solutions of [P]/[D] 0.8 and 0.6 exhibit the CD of Type III', which has a strong positive band at 541 nm, as seen in Fig. 3. Although the apparent CD is weak in this region, the actual molar ellipticity might be stronger than that shown, if correction is made for the amount of bound dye.

The absorption band of Acridine Orange at 470 nm becomes stronger when the dye concentration increases.[1] Its molar extinction coefficients are slightly influenced by the presence of the polymer for [P]/[D] 1 in very low pH region, which indicates very low binding of dye to the polymer. The CD spectra belong to Type II.

Dye–Low-molecular-weight Polymer Mixture. The low-molecular-weight polymer (E515) has a disordered conformation for all the pH in both absence[8] and presence[5] of Acridine Orange, and it interacts with the dye in a different way from the high-molecular-weight polymer. The induced CD spectra observed in the presence of the dye differ from each other, although the absorption spectra in the visible region are quite similar.

Absorption spectra indicate aggregation of dye in the solution of [P]/[D] 2 and more, as seen in Table 2. In the mixture of [P]/[D] 1, the species of dye changes at both sides of pH 5.4, while for [P]/[D] 0.8 the monomeric dye is dominantly present at all the pH examined. The hypochromism suggests the extensive binding of

TABLE 2.   THE pH REGION FOR THE INDUCTION OF CD OF VARIOUS TYPES

Polymer code	[P]/[D]	Absorption maximum		Type of CD				
		492 nm	470—450 nm	I	II'	II	III'	III
E602	25		4.0—11	4.0—6.7				
	2		5.8—11			6.8		6.9—10.0
	1	5.5—5.8	5.8—11			5.8		6.1— 9.9
	0.9	5.9—6.4	6.9—11				6.1—6.4	6.9— 9.9
	0.8	5.8—11					5.8—6.6	7.4— 9.9
E515	25		4.0—11			5.6—6.0		
	2		5.1—11			5.1—5.8		
	0.8	4.6—11			4.6—5.2			

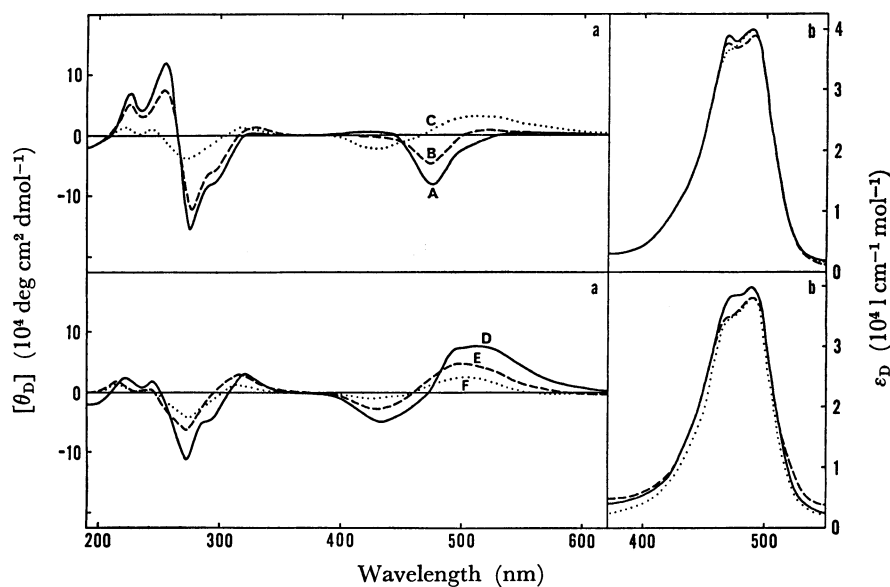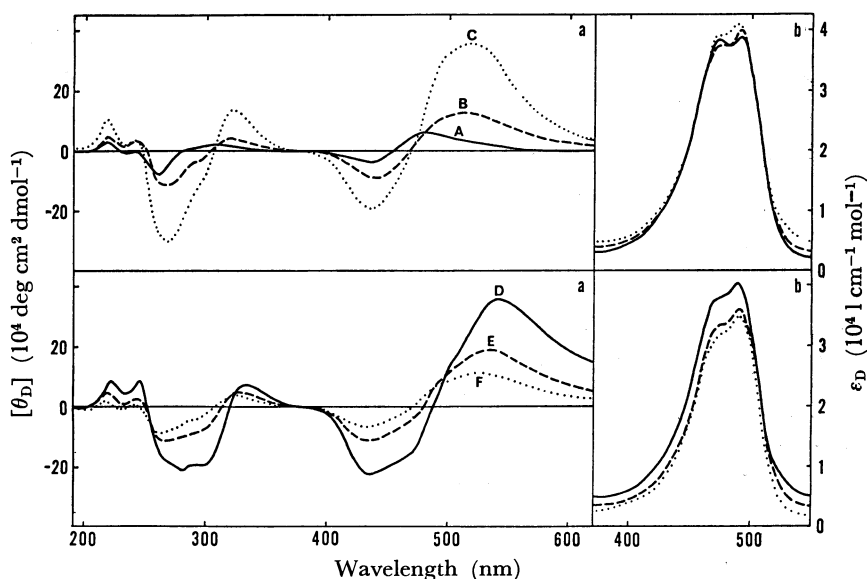

Fig. 2.   Circular dichroism (a) and absorption spectra (b) of Acridine Orange-poly(S-carboxymethyl-L-cysteine) mixture of [P]/[D] 1 in water at very low pH region. [D]=4.94×10⁻⁵ M.   A, pH 2.57; B, pH 2.68; C, pH 2.75; D, pH 2.91; E, pH 3.39; F, pH 3.81.

Fig. 3.   Circular dichroism (a) and absorption spectra (b) of Acridine Orange-poly(S-carboxymethyl-L-cysteine) mixture of [P]/[D] 0.8 in water at very low pH region. [D]=4.94×10⁻⁵ M. A, pH 2.14; B, pH 2.44; C, pH 3.09; D, pH 3.46; E, pH 3.61; F, pH 3.71.
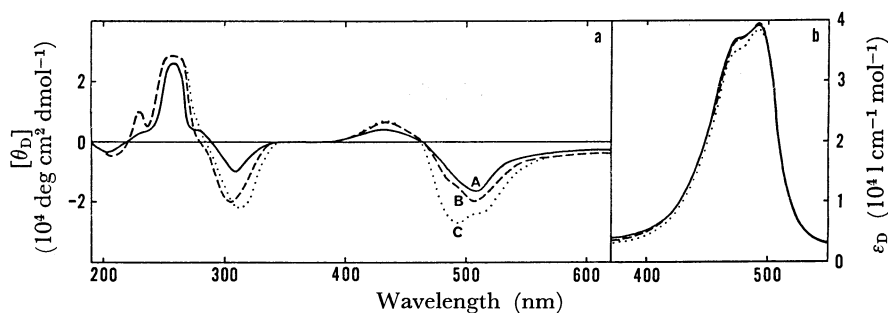
Fig. 4. Circular dichroism (a) and absorption spectra (b) of Acridine Orange-low-molecular-weight poly(S-carboxymethyl-L-cysteine) mixture of [P]/[D] 0.8 in water at very low pH region. [D]=4.94×10⁻⁵ M. A, pH 2.49; B, pH 3.13; C, pH 3.45.

dye to the polymer.

The CD is induced only at pH lower than about 6 for all the [P]/[D] values. The CD of the complexes formed at [P]/[D] higher than 1 belongs to Type II. The observed CD spectrum for [P]/[D] 0.8 consists of a positive band at 427 nm and a negative one at 533 nm, and it has been called Type II', as shown in Table 1.

The low-molecular-weight polymer (E515) yields precipitates at pH 3.51, 4.98, 4.80, and 4.36 for [P]/[D] 25, 2, 1, and 0.8, respectively. At very low pH, 2.0—3.7, the mixture of [P]/[D] 0.8 is soluble again and exhibits the CD of Type II' combined with Type II, as shown in Fig. 4. Since the absorption spectra in this region are very similar to those of free dye, indicating very few bound dye on the polymer, the observed CD has to be corrected for the amount of bound dye.

Discussion

Free Acridine Orange in water is dominantly in monomeric form at a concentration of 5×10^{-5} M, while it aggregates at high ionic strengths.[1,4] In the dye–polymer mixtures of low [P]/[D], monomeric form of dye is dominant over all the pH region. On the other hand, for higher [P]/[D], dimeric or higher aggregates of dye are formed on the polymer, since ionized carboxyl groups of the polymer and counter ions behave as the ionic atmosphere surrounding the dye molecules, making the dye molecules stack together.

The binding of dye to the polymer occurs primarily electrostatically, and a nitrogen atom of the cationic dye would be close to an ionized carboxyl group of the polymer, irrespective of whether the dye is monomeric or dimeric. In the absence of dye, the pH for the phase separation of polymer solution is lowered with decrease in concentration. For high [P]/[D], where the binding of dye is relatively low, the solubility of the polymer would not be influenced by such a binding. Thus the complex precipitates at low pH where the polymer is only slightly ionized. However, for [P]/[D] as low as 2, the phase separation occurs at pH as high as 5. When the divalent cationic dimer of Acridine Orange[1,2,10] binds to an ionized carboxyl group of the polymer, the charge on the carboxyl group turns from negative to positive. Thus the phase separation observed around pH 5 can be interpreted as the isoelectric precipitation of ampholytic complex. For [P]/

[D] lower than 2, the dye is mainly in monomeric form at low pH. When the monomeric dyes are bound to the polymer, the complex becomes electrically neutral at a certain pH and precipitates.

When greater amounts of cationic dyes are added to the polymer, or when [P]/[D] is lowered further, the pH of phase separation should be higher. However, the pH decreases for [P]/[D] less than 0.9. This could be caused by the decrease in concentration of the polymer. In the same way, more concentrated mixture for [P]/[D] 1 precipitates at slightly higher pH (Fig. 1), reflecting the solubility of the polymer itself.[8]

For the complexes of [P]/[D] 1 or less formed at low pH 4—2, the amount of bound dye is very small, since ionization of carboxyl groups is low and the cationic dye does not bind to uncharged carboxyl groups. However, the net charge of the complex would be positive and the complex becomes soluble. In this complex the polymer chain would be a random coil owing to the electric repulsion between attached positive charges. Thus it is unlikely that the observed CD is of Type I as observed for the β-conformation. The observed CD was either of Type II or Type III'. At very low pH around 2.5, the ionization of the polymer is too low to be soluble in solution.

The same discussion on the solubility behavior of the dye–polymer mixture would apply to that of the dye–low-molecular-weight polymer mixtures, and also to the dye–poly(L-glutamic acid) mixtures.[11]

References

1) V. Zanker, Z. Phys. Chem., 199, 225 (1952).
2) T. Kurcusev and U. P. Strauss, J. Am. Chem. Soc., 74, 3081 (1970).
3) R. E. Ballard and C. H. Park, J. Chem. Soc., A, 1970, 1340.
4) S. Ikeda and T. Imae, Biopolymers, 10, 1743 (1971).
5) T. Imae and S. Ikeda, Biopolymers, 14, 1213 (1975).
6) S. Ikeda and T. Imae, Polymer J., 4, 301 (1973).
7) T. Imae, Polymer J., 9, No. 6 (1977) in press.
8) S. Ikeda, Biopolymers, 5, 359 (1967).
9) J. Y. Cassim and J. T. Yang, Biochemistry, 8, 1947 (1969).
10) M. E. Lamm and D. M. Neville, J. Phys. Chem., 69, 3872 (1965).
11) T. Imae and S. Ikeda, Biopolymers, 15, 1655 (1976).