BIOPOLYMERS VOL. 14, 1213–1221 (1975)

On the Various Types of Circular Dichroism Induced on Acridine Orange Bound to Poly(*S*-carboxymethyl-L-cysteine)

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Synopsis

Circular dichroism and absorption spectra are measured on mixed solutions of acridine orange and poly(*S*-carboxymethyl-L-cysteine) at different pH and P/D mixing ratios. The observed circular dichroism spectra are classified into several types, mainly based on the number and sign of circular dichroic bands in the visible region. Three of them are associated with the absorption spectra characteristic of dimeric dye or higher aggregates of dye. Type I is observed with solutions, of which the pH is acid and P/D is higher than 4, and it has an unsymmetrical pair of positive and negative dichroic bands at 470 and 430 nm. This type is induced on the dye bound to the polymer in the β-conformation. Types II and III are considered to be characteristic of randomly coiled polymers. Type II is exhibited by solutions of P/D higher than 1 at pH 5–7 and has two dichroic bands around the same wavelengths as Type I but with opposite signs and an additional positive band at 560 nm. Type III, shown by solutions of P/D 2–0.6 at pH 6–10.5, has three dichroic bands around the same wavelengths as Type II but with signs opposite to it. The other two types of circular dichroism, induced for the solutions of P/D less than 1 at slightly acid pH, are associated with the absorption spectra of monomeric dye and are observed with disordered or randomly coiled polymer. They have a pair of dichroic bands at 540 and 425 nm, and the signs of these bands are opposite to each other in these two types.

INTRODUCTION

In a previous paper,[1] we noted that acridine orange is induced to form characteristic circular dichroism (CD) absorption bands when it is mixed with β-form poly(*S*-carboxymethyl-L-cysteine) in solution. We presented a possible model for the mode of binding of acridine orange on the polypeptide and explained the sign and magnitude of the observed CD bands.[2] However, the induction of this kind of CD was limited to the case when the pH was acid and the mixing ratio of polymer residue to dye, P/D, was higher than about 4.

In the present paper, we will report various types of CD induced on acridine orange in the presence of poly(*S*-carboxymethyl-L-cysteine), which are observed at different pH and P/D and with polymers of different molecular weights.

EXPERIMENTAL

Materials

Samples of poly(*S*-carboxymethyl-L-cysteine) were the same as previously used.[3] A sample of code E602 was investigated in detail and another sample of code E515 was used as a low-molecular-weight polymer. Acridine orange was purified from the commercial dye, as previously described.[1,4]

In all the experiments, the dye concentration was kept constant at 4.94×10^{-5} *M*, and the mixing ratio, P/D, was chosen as 25, 4, 2, 1, 0.9, 0.8, and 0.6. The ionic strength was adjusted by the addition of NaCl into the stock solution of the polymer, if necessary. The mixed solution of dye and polymer was prepared by adding a dye solution to the stock solution of the polymer, and then pH was adjusted by HCl or NaOH, followed by dilution to a desired dye concentration. The spectra were measured on the solution kept overnight after its preparation.

Apparatus

The pH of the solution was measured with a Hitachi-Horiba F5-X pH-meter. Absorption spectra were recorded on a Hitachi 323 recording spectrophotometer over the region from 600 to 350 nm. CD spectra were recorded on a Jasco J-20 circular dichrometer over the region from 620 to 185 nm, which was calibrated by aqueous solution of d-10-camphor sulfonic acid.[5] Measurements were performed at room temperature (25°C). The cell path length was chosen as 5 mm for the absorption measurements and 10 mm for the CD measurements. For the determination of CD at the far-ultraviolet region, a cell of 1-mm path length was used, if necessary.

All the data presented below are reduced on the molar basis of total concentration of added dye, and are expressed by the molar extinction coefficient ϵ_D and the molar ellipticity $[\theta_D]$. The ultraviolet CD is sometimes reduced on the residue basis of peptide groups, and is expressed by $[\theta_P]$.

RESULTS

The absorption spectra of acridine orange in water are not dependent on pH over the region pH 2–9. They are characterized by the two absorption bands at 492 and 470 nm in the visible region, when the dye concentration is about 5×10^{-5} *M*. The two bands have been ascribed to the monomer and dimer of the dye,[4] and their molar extinction coefficients are about 42,000 and 36,000, respectively, around this concentration. When the dye concentration is much higher than this, the absorption band shifts further to the blue to 450 nm, together with the change in molar extinction coefficients. This may be attributed to the formation of higher aggregates of dye or aggregates of dimeric dye.

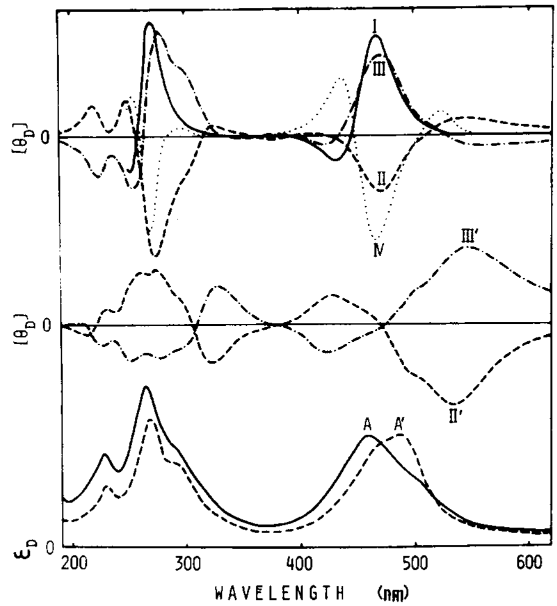

Fig. 1. Schematic representation of various types of circular dichroism and absorption spectra of acridine-orange–polymer system.

When poly(*S*-carboxymethyl-L-cysteine) is added to a solution of acridine orange of the concentration 5×10^{-5} *M*, blue shift and hypochromism often occur on the absorption spectra. The blue shift indicates that the dye has formed dimers or higher aggregates; the hypochromism suggests the binding of dye with the polymer or the formation of a complex of dye with the polymer.

Absorption spectra of acridine orange bound to the polymer are illustrated in Figure 1. For the solutions of P/D higher than 2, the main absorption band is located around 450 nm over the entire pH region, suggesting the formation of highly aggregated forms of dye.[1,4] These spectra are designated as *A*. For the solutions of P/D 0.8 and 0.6, however, the band at 490 nm is always stronger than that at 470 nm, indicating that the monomeric dye is dominant. These spectra are *A'* in Figure 1.

The molar extinction coefficient of the main band does not appreciably change with pH for a given P/D value. The observed hypochromism amounts to about 25 to 50%, suggesting the extensive binding of dye to the polymer. For P/D lower than 0.8, the extinction coefficient increases and thus the binding of dye decreases.

On the other hand, the CD spectra vary remarkably with the changes in pH as well as in P/D. By surveying a number of CD curves observed at different pH and P/D, we have classified these CD spectra into several fundamental types, mainly based on the number and signs of visible CD bands. We will be able to construct any of the observed CD spectra by combining two of these types or to assign it to one of them. Figure 1 illustrates these fundamental types of CD spectra.

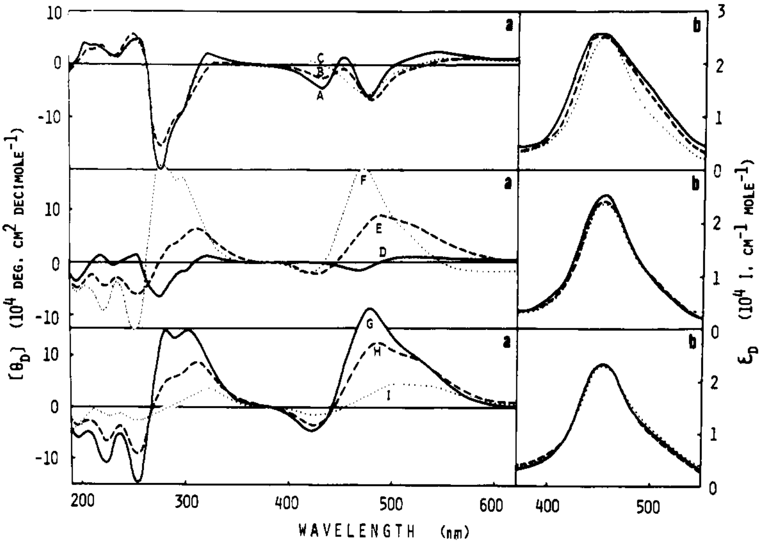

Fig. 2. Circular dichroism *a* and absorption spectra *b* of acridine-orange–polymer system of P/D 2 in water. $[D] = 4.94 \times 10^{-5}$ *M*. *A*—pH 5.84; *B*—pH 6.25; *C*—pH 6.80; *D*—pH 6.88; *E*—pH 6.91; *F*—pH 7.43; *G*—pH 8.23; *H*—pH 8.71; *I*—pH 9.98.

When P/D is higher than 4, the CD is induced around the dye absorption bands only at the acid pH, 7.0–3.5. A pair of positive and negative bands are manifest around each of the visible and ultraviolet absorption bands, and this is given in Figure 1 as Type I. The CD is identical with that previously reported.[1]

As P/D is lowered below 4, marked changes in CD are observed. For the solution of P/D 2 or 1 at acid pH, the observed CD around each absorption band consists of two paired bands with signs opposite to Type I and an additional weak positive band at the longer wavelength side. The CD is shown in Figure 1 as Type II and can also be seen as curve *C* of Figure 2.

When P/D is lower than 4, CD is induced even at the neutral or alkaline pH. Typical CD spectra are curve *F* of Figure 2. They have three CD bands at each absorption region and their signs are opposite to those of Type II. This is given in Figure 1 as Type III.

As is also seen in Figure 2, all the above CD types, I, II, and III, are associated with the dimeric dye as characterized by the absorption band at 470 nm or lower, or by the spectra *A*.

While Type-III CD is observed at alkaline pH for low P/D, the other type of CD emerges with the solutions of P/D less than 0.9 at acid pH. As can be seen in Figure 3, the positive bands shift to the red with decreasing pH. Since the corresponding absorption spectra indicate that the monomeric dye species is present or the spectrum is *A′*, but the CD spectra are similar to Type III, we designate the CD as Type III′ and also show it in Figure 1.

The effect of added salt on the interaction of dye with the polymer is rather remarkable for the solution of P/D 1 around neutral pH. When

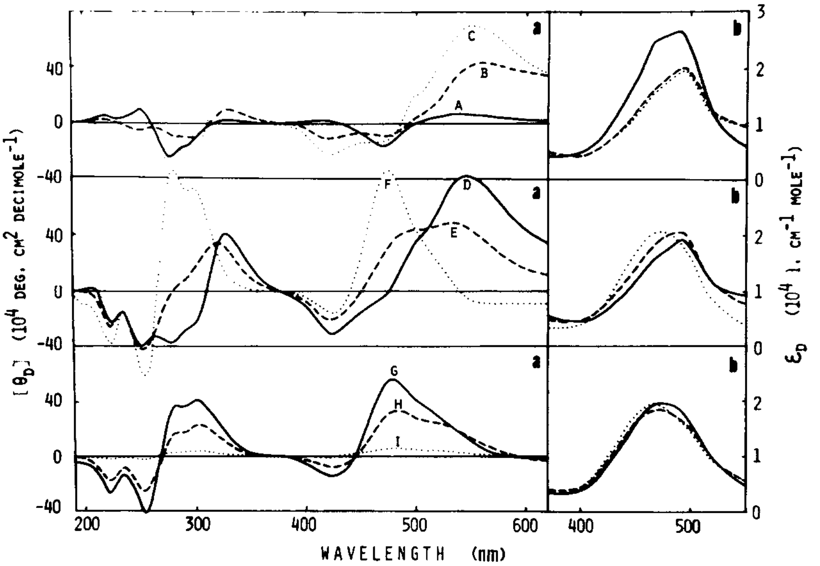

Fig. 3. Circular dichroism *a* and absorption spectra *b* of acridine-orange–polymer system of P/D 0.9 in water. $[D] = 4.94 \times 10^{-5}\ M$. *A*—pH 5.90; *B*—pH 6.12; *C*—pH 6.16; *D*—pH 6.26; *E*—pH 6.41; *F*—pH 6.94; *G*—pH 7.26; *H*—pH 9.43; *I*—pH 9.87.

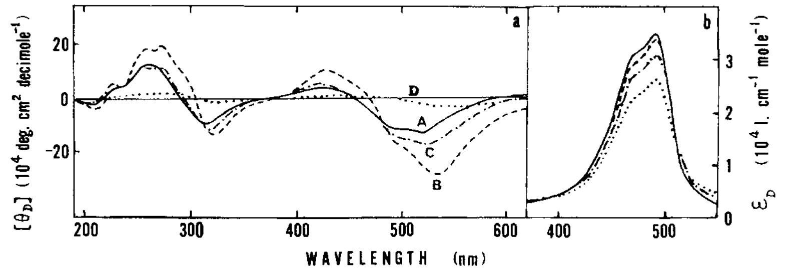

Fig. 4. Circular dichroism *a* and absorption spectra *b* of acridine-orange–low-molecular-weight-polymer system of P/D 0.8 in water. $[D] = 4.94 \times 10^{-5}\ M$. *A*—pH 4.60; *B*—pH 4.84; *C*—pH 5.04; *D*—pH 5.24.

0.002 *M* NaCl is present, the visible absorption band simply shifts slightly to red, but the molar ellipticity of Type-III CD reduces to about 1/3 of that in the absence of added salt. The presence of 0.2 *M* NaCl makes the absorption spectra very similar to those of free dye in 0.2 *M* NaCl, and it causes the CD to disappear at the entire pH regions examined.

The interaction of acridine orange with the low-molecular-weight polymer (E515) is found to be different from that with the high-molecular-weight polymer surveyed above. This was partly noted previously.[1]

For high P/D ratios, the absorption spectra indicate the formation of dye aggregates and the CD spectra are close to Type II. However, with decreasing P/D lower than 2, the aggregation of dye becomes suppressed. Figure 4 shows the CD spectra as well as the absorption spectra for the

TABLE I
Types of Circular Dichroism

	I	II	III	II′	III′
Visible CD bands	—	560 (+)	557 (−)	533 (−)	545 (+)
(nm)	466 (+)	472 (−)	470 (+)	—	—
	433 (−)	415 (+)	422 (−)	427 (+)	421 (−)
Unit of bound dye	Dimer	Dimer	Dimer	Monomer	Monomer
Polymer conformation	β	Random coil	Random coil	(Random coil)	(Random coil)
P/D	>4	>1	2–0.6	1–0.8	0.9–0.6
pH	3.5–7	5–7	6–10.5	4.5–5.5	5–7

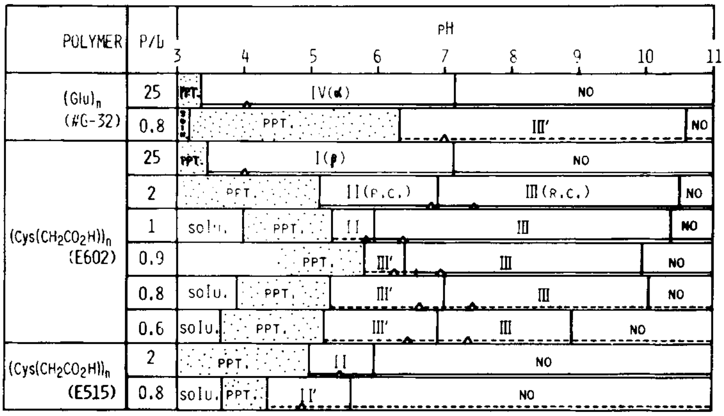

Fig. 5. Behavior of acridine-orange–polypeptide systems in water. $[D] = 4.94 \times 10^{-5}$ M. Roman numeral represents the type of CD spectra. Solid horizontal line indicates the presence of dimeric dye and dashed horizontal line means the dominant presence of monomeric dye. α, β, and R.C. in parentheses represent the polymer conformations, α-helix, β-conformation, and random coil, respectively.

solutions of P/D 0.8. The CD spectra are of those corresponding to Type II but the band positions are red-shifted generally, since the main species of dye in concern is monomeric. The CD is given in Figure 1 as Type II′. It is noted that no CD is induced at pH higher than about 6 for all P/D ratios.

In summary, Figure 5 indicates the ranges of pH and P/D values, at which each type of CD spectrum is observed. It also shows the dye species, whether it is monomeric or dimeric, at those ranges, as derived from the type of absorption spectra. Table I lists the position of visible CD bands and the range of appearance of various CD types.

DISCUSSION

Conformation of the Polymer Chain

The polypeptide chain conformation may be approximately deduced from the CD bands at 200 and 225 nm in the presence of acridine orange. The

CD associated with the π-π^* transition of peptide groups at 200 nm changes its sign from positive to negative when the polymer undergoes a transition from the β-conformation to the random coil.[6-8]

For the solutions of P/D 25 at pH 4.03, a positive CD band with $[\theta_P]_{200}$ = 31,000 is observed, which clearly means that the polymer is in the β-conformation. Upon raising the pH, the sign of the CD bands changes from positive to negative, indicating the occurrence of transition from the β-conformation to the random coil. At pH 6.86, a negative band with $[\theta_P]_{200}$ = 15,000 is attained. The corresponding change is seen in the magnitude of the negative band at 225 nm.

For P/D 4, the CD band with $[\theta_P]_{200}$ = 13,000 suggests low content of β-structure even at the most acid pH, 4.77, and thus it seems likely that the transition pH is higher for P/D 4 than for P/D 25. As P/D is lowered further, the CD associated with the π-π^* transition is less clear, but the pH for the β-coil transition seems to be higher. The latter is opposite to the behavior of the polymer in the absence of acridine orange, as observed when the polymer concentration is varied.

From these results we may imagine that the extensive binding of dye to the polymer would influence the polymer conformation at a given pH. However, we may also conclude that, while CD of Type I is induced on the dye bound to the polymer in the β-conformation, the CD of Types II and III is induced on the dye bound to the randomly coiled polymer.

In the dye–low-molecular-weight-polymer system of P/D 25, the polymer conformation is disordered even at pH 3.99. This result is consistent with the previous results that the polymer cannot assume the β-conformation but is in the disordered conformation.

Types of Circular Dichroism and the Structure of Dye–Polymer Complexes

Figure 5 indicates the ranges of pH and P/D for the appearance of different types of CD in the system of acridine orange and poly(*S*-carboxymethyl-L-cysteine). It also includes the CD of acridine orange bound to poly(L-glutamic acid).[9-11] Table I lists the details of visible CD bands.

It is evident from the correlation of CD with absorption spectra that CD of Types I, II, and III (and IV) is associated with dimeric or higher aggregated forms of dye, while CD of Types II′ and III′ is induced on the monomeric dye. For P/D 0.8 and 0.6 at alkaline pH, the CD (Type III) is associated with the dimeric dye, while the absorption indicates the presence of monomeric dye. This is due to the absorption of free monomeric dye present in large excess.

The polarization direction of two main absorption bands, visible and ultraviolet, of acridine orange has been assigned as parallel to the longer axis of the dye molecular plane.[12,13] Since a structure of antiparallel stacked type has been proposed[4] and accepted[14,15] for the dimeric dye molecule, the polarization direction of the bands remains unaltered upon dimerization of dye. That is, the monomeric level at 492 nm and the dimeric level at 470 nm of the visible absorption band should have a common polarization direction.

Based on these electronic properties of acridine orange, we may speculate on the structure of complexes that exhibit different types of CD. Type-I CD is induced on the aggregates of dimeric dye bound to the β-form polymer and was examined in detail previously.[1,2] The complex has the structure in which the dimeric dye molecules are bound to an extended polypeptide chain in the β-conformation and stack together up and down along the chain. The molecular planes of the dye are almost perpendicular to the direction of polymer chain but slightly tilted from it in such a way that they form a twofold right-handed helix. This structure of the complex was also confirmed to hold for the complexes of two diaminoacridines, proflavine and acridine yellow, formed with the same polymer in the β-conformation.[16]

Type-III CD is induced on dimeric dye bound to the random-coil polymer at high ionization and is characteristic of low P/D ratios. Almost all the ionized carboxyl groups would be occupied by the bulky dimeric dye cations. The high charge density and bulky bound dye would cause the polymer chain to extend owing to the electrostatic repulsion and steric hindrance. Then the structure of the complex would be similar to that found in the dye–β-form-polymer complex.

It was demonstrated[17] that a linear alternating array of chromophores induces a pair of positive and negative CD bands around each absorption band, if the polarization of chromophores forms a helical array. The sign and relative location of the paired bands are determined by the sense of the polarization helix and a sum of interaction potentials between chromophores across the center line of the array. If the dye–polymer complex has the structure as assumed above, in which the polarization of dye is almost perpendicular to the extended polymer chain, all the interaction potentials across the polymer chain are negative. To conform with the observed sign and relative location of the paired bands at 470 and 422 nm in the visible region, the sense of the polarization helix must be right-handed.

The presence of an additional CD band at 557 nm indicates, however, that all the dye molecules do not always form a perfect linear alternating stack, but some of them are arranged also in the other configurations, each of which would be regarded as a part of various helices. Then the extra band should be a longer wavelength limb of the pair of dichroic bands, which are characteristic of general helices other than twofold helix.[17–19] It should arise from the contribution of incident light parallel to the helix axis.[18,19]

Type-II CD is also induced on dimeric dye bound to the random-coil polymer, but at low ionization. The polymer chain would be more compact and the binding of dye would be less. The bound dye is localized owing to the stacking tendency of the dye and the tendency of even distribution of charged sites.

Types-II′ and III′ CD are considered to be counterparts of Types II and III, respectively, and are associated with the monomeric dye. Type-III′ CD is likely to be concerned with the random-coil polymer, since it is observed at only slightly acid pH. Type-II′ CD is associated with the low-molecular-weight polymer, which remains in the disordered conformation.

Although calculations of CD of random-coil polypeptides have been published by several workers,[20–23] their validity has not always been assured definitely. Until the theory of CD of random coil is further elaborated, we must await further consideration of the structure and CD of the dye–random-coil-polymer complexes.

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Received October 4, 1974
Accepted December 17, 1974