Kinetic Studies of the Aggregation of Acridine Orange onto Poly-(α,L-glutamic acid)

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The aggregation process of acridine orange (AO) onto the helical poly-α,L-glutamic acid (PLGA) was studied by means of a stopped-flow and rapid-scan spectrophotometer. After the flow stop, a rapid spectral change was observed in which the α-band and the β-band fell, and the γ-band and the polymer band rose. A mechanism of the process was proposed by which after a rapid nucleative process, AO molecules are cooperatively bound to form a dimer and that then the dimer grows to the oligomer and to the polymer of AO which has the form of a super-helix around the core of the α-helix of PLGA.

The main feature of the binding of a number of cationic dyes to polyanions is the so-called metachromasia,[1-3] an effect which has been recognized as due to different binding modes of the dye molecules exhibiting different absorption spectra in the visible region. The absorption bands show that the dye molecules bind to the polyanion in their monomeric forms, or interact with each other on the polyanion to form dimers or aggregates similar to those existing in concentrated aqueous solutions.[4-7] Various theories with regard to the phenomena have been proposed and have been rigorously tested by studying the equilibria[8-19] and the dynamic properties[20-26] of the dye-polymer interaction in aqueous solutions. So far, however, the phenomena have not been able to be interpreted in terms of a generally accepted mechanism.

The interaction of acridine orange (AO) with poly-α,L-glutamic acid (PLGA) has been extensively studied.[18-20] Since PLGA exists predominantly as the α-helix in aqueous solution below about pH 5 and as a random coil above pH 7,[27-28] the role of the structure of the polyanion in the dye-PLGA interaction can be discussed.

Hammes and Hubbard[20] reported kinetic studies of the interaction of AO and PLGA at pH 4.7 and pH 7.5. They proposed a mechanism in which an AO binds very rapidly to a site on the polymer, followed by a slower intramolecular rearrangement. Their results suggested that the same general mechanism appears to be operative for both the helical and random coil forms of PLGA. On the other band, Schwartz and his co-workers[23] proposed a cooperative binding mechanism for the association of AO with PLGA on the basis of thermodynamic and kinetic analyses. Since their measurements, however, followed only the decay of the absorbance of the α-band (monomeric form of AO), the mechanism could not interpret the processes of the formation of the other aggregates.

The associations of AO with itself and with PLGA are both very rapid processes, so the temperature-jump method has been used in most kinetic measurements.[20,23] This method, however, requires an ionic strength of about 0.1 M for the maintenance of the resistance of the solution at an appropriate value. The overall association constant for the AO and PLGA complexes has been reported to be markedly decreased as the ionic strength is increased.[11-14] Because of the above facts, the strong binding processes of the complex cannot be observed in the conventional temperature-jump method. Although the time of observation is limited by its relatively long dead time (slightly less than 1 ms), the stopped-flow method has an advantage in its applicability to a wide range of salt concentrations.[25]

A rapid-scan spectrophotometer was used for the measurement of the change in the spectral pattern after the flow stop. The fall and rise of several spectral bands of the AO and PLGA complexes could successfully be observed by means of this apparatus. In this investigation, the aggregation process of AO onto PLGA was studied in the various ionic atmosphere to clarify the mechanism.

Experimental

The AO was purified according to the following method. An aqueous solution of commercial AO (2 g) purchased from the Merck Co. was titrated with 0.1 M NaOH to pH 10.5. The suspension was then filtered, and the residue was washed with 100 ml of 3×10^{-3} M EDTA. This procedure was repeated. The residue was then dried at 100 °C and dissolved in absolute ethanol. The solution was neutralized with ethanolic HCl, refluxed for 1 hr, and filtered to remove the NaCl formed. The filtrate was passed through an $8\times$ 40 cm acid-washed alumina column in ethanol. The elute comprising the main fraction was then filtered to remove any alumina particles present. The AO–HCl was precipitated from the filtrate by the addition of ether, collected by filtration, and dried under a vacuum. The melting point of the compound was sharp at 181 °C (Ref. 5, 180—181 °C), and the molar extinction at the absorption maximum (492 nm) extrapolated to zero concentration is 6.3×10^4 (6.1×10^4 after Zanker[5]). Generally we prepared a stock solution of 1 mM AO by weighing it in a dry polyethylene bottle. The concentration of AO was determined from the absorbance at 492 nm according to the extrapolated equation[11] for a dilute solution.

The sodium poly-α,L-glutamate was purchased from the Protein Research Foundation (Osaka); it had a weight-average molecular weight of 60,000. The poly-α,L-glutamic acid solution was made by the deionization of the salt with 0.1 M HCl and was dialyzed against frequent changes of the deionized distilled water.[27] Each solution of AO and PLGA was prepared in 2 mM sodium acetate-acetic acid (pH 4.7), adding sodium chloride to adjust the ionic strength to the chosen value. The final pH was maintained approximately

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at pH 4.7 throughout the experiments. In this pH region, PLGA exists predominantly as the α-helix in an aqueous solution.[27,28] All the experiments were performed at 25 °C.

The optical absorption spectra were taken with a recording spectrophotometer (Hitachi EPS-3T). The kinetics of the AO-PLGA reaction was studied using a rapid-scan spectrophotometer equipped with a stopped-flow apparatus (UNION RA-1300). After equal volumes of AO and PLGA solution had been rapidly mixed, the spectral patterns were measured by means of an image detector with a maximum speed of 300 nm/ms and were memorized by a transient recorder. The device contains five channel transient memories. Each digitizes a transient signal with an 8-bit resolution and stores it in a 512-word MOS shift-resister memory. Its sweep time can be varied between 1 ms and 10 s. An analog replica of the original can be recorded on a X-Y recorder as spectral patterns of the transmittance, absorbance, or time resolution difference spectra.

For the kinetic measurements, an absorption or fluorescence stopped-flow apparatus was used. The fluorescence was observed from the right angle to the excitation beam (492 nm), through an interference filter of 552 nm.

Results and Discussion

AO aggregates in an aqueous solution.[5] It is thought that there are at least three types of species; they may be defined as monomers, dimers, and oligomers[5-7] and their absorption bands are located at 492 nm(α-band), 464 nm(β-band), and 445 nm(γ-band), respectively. When AO is mixed with a polyanion in a dilute aqueous solution, the color of either the monomer or the aggregates may appear, depending upon the ionic strength, the pH, and the relative amounts of polymer and dye (P/D ratio).[8-18]

Figure 1 shows the visible absorption spectra of the AO (2.0×10^{-5} M) and PLGA complex at the P/D ratio of 10 while the ionic strength is changed. As the ionic strength is lowered, the shape of the spectrum is continuously changing; that is, the absorbance of the α-band decreases relative to that of β-band, and both the bands fall, while the absorbance of the γ-band and the band around 535 nm rise.

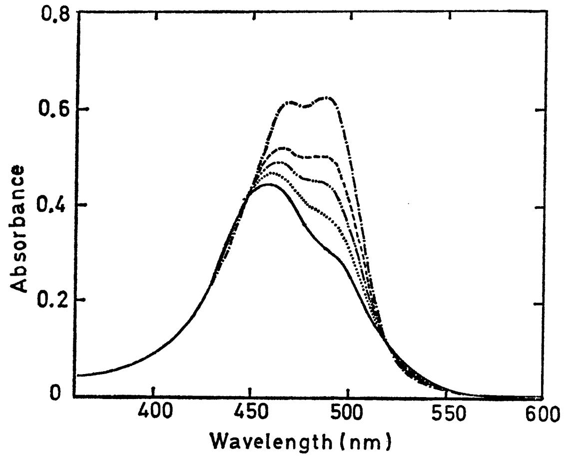

Fig. 1. Effect of ionic strength upon visible absorption spectra of AO-PLGA solutions. Solvent is 0.002 M sodium acetate-acetic acid (pH 4.7) and the ionic strength is adjusted by NaCl. The molarities of AO and PLGA are 2.0×10^{-5} M and 2.0×10^{-4} M, respectively. —— =0.005, ······ =0.01, —··— =0.05, ---- =0.10, —·— =0.20.

The results suggest that the aggregation of dye was brought about at a low ionic strength.

The formation of these aggregates upon mixing of AO with PLGA at a low ionic strength was measured with the rapid-scan spectrophotometer. Figure 2(a) shows the results on the rapid spectral change in the reaction mixture at the times of 0, 10, 100, and 400 ms after the flow stop, the scanning speed being constant at 150 nm per 1 ms. The abrupt change in the spectra before and after the flow stop shows that almost all of the reaction occurred during the dead time (about 1 ms). After the flow stop, the α-band and the β-band fall, but the change in the other bands is not clear. To clarify the change in these absorption bands, we observed the time resolution difference spectra, which were obtained using the analog device by subtracting the absorbance at an appropriate time from the corresponding absorbance immediately after the flow stop. The results are illustrated in Fig. 2(b); they show that the α-band and β-band fall but γ-band and the band around 535 nm appear slightly. The above facts suggest that the reaction between AO and PLGA does not proceed in a single step, rather, spectrally different steps may be involved.

The mechanism of the binding processes of AO

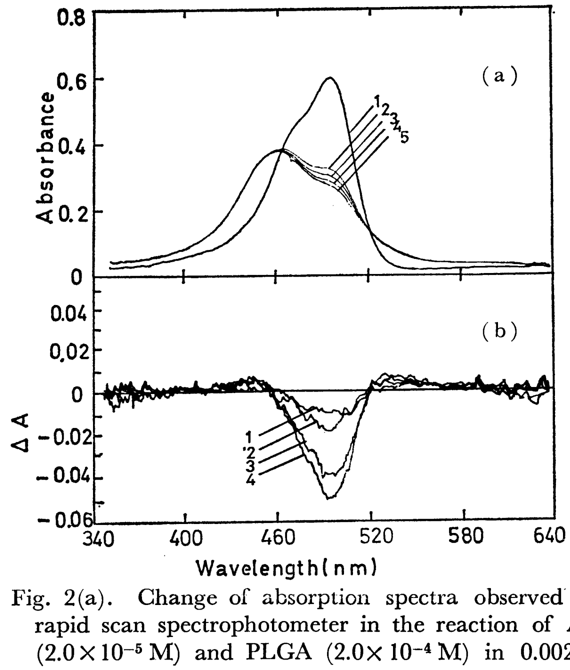

Fig. 2(a). Change of absorption spectra observed by rapid scan spectrophotometer in the reaction of AO (2.0×10^{-5} M) and PLGA (2.0×10^{-4} M) in 0.002 M acetate buffer (pH 4.7) at 25 °C. Scan speed is 150 nm/ms.

1. free AO (2.0×10^{-5} M), 2. immediately after the flow stop, 3. 10 ms after, 4. 100 ms after, 5. 400 ms after.

Fig. 2(b). Time resolution difference spectra observed by rapid scan spectrophotometer. The reaction conditions are the same as those in Fig. 2(a). ΔA is absorbance at appropriate time minus the corresponding absorbance immediately after the flow stop.

1. 4 ms after the flow stop, 2. 10 ms after, 3. 100 ms after, 4. 400 ms after.

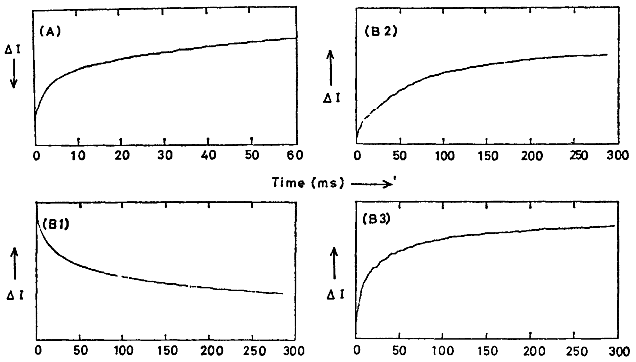

Fig. 3. The time course of AO-PLGA interaction observed by stopped-flow method. The figures are records of analogs replica from transient memories. The reaction conditions are the same as those in Fig. 2(a) and Fig. 2(b).
(A): Fluorescence emission observed at 552 nm through an interference filter (decrease upward).
(B): Change of absorbance of each absorption band. The vertical axis corresponding to the change in optical density which increase upwards.
(B1): 490 nm (α-, β-band).
(B2): 435 nm (γ-band).
(B3): 535 nm.

onto PLGA was studied by means of the stopped-flow apparatus. The decrease in the AO monomer in the species was observed by means of fluorescence quenching,[5)] and the formation of the aggregates was followed by the change in the absorbance of the α-band (490 nm), the β-band (435 nm), and the band around 535 nm. Figure 3 shows a replica of the transient-memory recordings of some typical kinetic data. For each absorption band and emission band, a plot of the logarithms of the amplitude of the light intensity change *vs.* time was curved. The non-linear relaxation spectra thus obtained suggest that a cooperative binding of the dye over the polymer or some aggregation processes may take place.

The mean relaxation time (τ^*) of the non-linear relaxation spectra can be obtained in a good approximation during an initial period, with more or less appreciable deviations later on. Accordingly, τ^* was conveniently determind from the pertinent experimental curve.

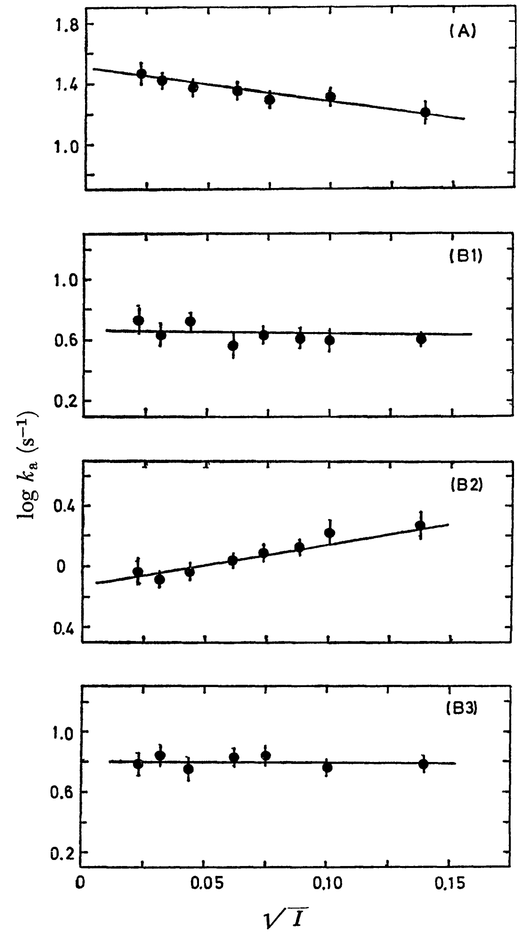

Fig. 4. Plots of log k_a against the square root of the ionic strength obtained with each spectral band as shown in Fig. 3. The curve number and the reaction conditions except ionic strength are the same as those in Fig. 3.

To make the aggregation processes clear, the effect of the salt concentration on the relaxation time of each spectral band was studied, for the association is thought to involve electrostatic interaction between the oppositely charged dye and residue of the polymer in addition to nonelectrostatic interaction. According to the transition-state theory and the Debye-Hückel theory,[30)] the relation between the rate and the ionic strength is given for dilute solutions by;

$$\log k = \log k_0 + 1.02 \cdot Z_a Z_b \sqrt{I} \qquad (1)$$

where Z_a and Z_b are the numbers of the charges on the ion and where k and k_0 are the rate constants at a finite and at an infinite dilution respectively in a given solvent. The mechanism for the overall reaction is not clear now, so we assume that the apparent rate constant is approximately linear to the reciprocal relaxation time at a constant concentration. Equation (1) predicts a linear relationship if log k_a is plotted against the square root of the ionic strength with a slope proportional to the Z_aZ_b product. Figure 4 shows plots of the apparent rate constants over the range of ionic strength from 5.0×10^{-4} to 2.0×10^{-2}. The results of fluorescence observation give a negative value for the Z_aZ_b product. This shows that the reactants are oppositely charged. As AO is ionized to be cationic at pH 4.7, with its pK_a of 10.45,[5)] the other reactant may be the negatively charged site of PLGA. The charge of the site of PLGA can be derived -1.3 ± 0.4 from the values of the product of $Z_a{}^* Z_b = -1.6 \pm 0.5$ and the effective charge[††] of $Z_a{}^* = +1.2$. The result is consistent with the fact that the side-chain of PLGA is the mononegative

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[††] The differnce in binding affinity to the charged site of PLGA between the monomer and the dimer of the AO cation is not known. Assuming that Coulombic force may play an important role in the binding force, the effective charge, $Z_a{}^*$, of the free AO in the reaction mixture can be estimated for a given reaction. At concentration of AO used (2.0×10^{-5} M), the fraction of the dimer in free AO is estimated to be *ca.* 10%.

anion of the carboxyl group at pH 4.7.

The rate for the decrease in the absorbance of α-band was independent of the ionic strength. This suggests that two or more cationic AO molecules were associated at the occupied site of PLGA, where the negatively charged carboxyl group of the polypeptide had been neutralized by a cationic AO molecule and formed a dimer or oligomers of AO outside the complex. The close resemblance in the magnitude of the rates and the Z_aZ_b products between the rise of the band around 535 nm and the fall of the α-band and β-band indicates that the process of the decay of dimeric AO is the rate-limiting step for the rise of the band around 535 nm. Therefore, the appearance of the band around 535 nm may be due to formation of polymeric AO along the axis of the α-helix of PLGA.

The positive sign of Z_aZ_b observed at the γ-band suggests that both of the reactants are similarly charged ions. It seems reasonable to consider that the cationic AO molecules bind to the similarly charged site of PLGA, where more than two cations have been bound to a carboxylate anion of the side chain of the polypeptide. The effective charge of the Z_a^* of the free AO in the reaction mixture can be estimated as $+0.9$, as the similarly charged ions associate with each other in this process. As the value of the $Z_a^*Z_b$ product was given as 3.2 ± 0.4 from the results, the charge of the binding site of PLGA can be estimated as 3.5 ± 0.5. These facts show that the residue of PLGA is occupied by four or five cationic AO molecules and that the oligomer bound in the outside of the helical PLGA.

Although the aggregation theory successfully explains the monomer-dimer equilibrium, the nature of the higher aggregates is not yet clear. Schwartz[7] assumed that the spectra of the γ-band is independent of the length of the oligomer. The present work shows that the γ-band appears with the association of five or six AO molecules and that the formation of the further aggregates gives rise to a polymer band around 535 nm. This polymer band of AO has also been confirmed by the CD study of Hatano *et al.*[19]

The results suggest that the primary process of the reaction mechanism is one in which the AO cation binds very rapidly to the carboxyl anion of PLGA and the charge of the binding site is neutralized. This process may be followed by the cooperative binding of a cationic AO molecule to an unoccupied site adjacent to the occupied site of PLGA and by the formation of an oligomer and polymer of AO onto the helical PLGA.

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