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# Equilibrium and Kinetic Studies of the Dimerization of Acridine Orange and Its 10-Alkyl Derivatives

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The thermodynamics and kinetics of the dimerization of Acridine Orange (AO, 3,6-bis(dimethylamino)-acridinium chloride) and its 10-alkyl derivatives in 0.1 M NaCl solution have been studied by spectrophotometry and temperature-jump method. Dimerization constants, which were determined spectrophotometrically, become large with an increase in the length of the introduced alkyl chain; this behavior is the same as that in water and a dilute HCl solution. The association and dissociation rate constants determined from the temperature-jump measurements also depend on the length of the alkyl chain. Especially, the rate constants for methyl-AO are unexpectedly small. Both the effect of added salt on the thermodynamic parameters and the kinetic results are discussed in view of the structure of hydration water surrounding dye molecules. $^1$H NMR data for AO and methyl-AO suggest that the planes of two component molecules in a dimer are parallel to each other. This result is consistent with the spectrophotometric and kinetic data.

Aggregation of metachromatic dyes in aqueous solution has extensively been studied as the most fundamental model system for the self-aggregation of biomolecules and the interaction between small molecules and biological macromolecules. Among these, Acridine Orange (AO) has particularly attracted many interests[1—15] because of its bacteriostatic and mutagenic activities as well as the ability of binding with nucleic acids.

In recent years, equilibrium studies on the dimerization of 10-alkyl derivatives of AO have been performed to elucidate the role of hydrophobic interaction.[11,13] As a result, the dimerization constant, the enthalpy and entropy changes for the dimerization were found to become large with an increase in the length of the introduced alkyl chain; the behavior has been interpreted in terms of the enhanced hydrophobic interactions. Kinetic studies for AO[3,7], 10-octyl- and 10-decyl-AO[8] have also shown that the greater stabilities of dimer for the long alkyl chain derivatives are realized by the small dissociation rate constants.

However, the mechanism of the dimerization is still not completely elucidated. The present paper describes the details of the thermodynamics and kinetics of the dimerization, using various 10-alkyl derivatives of AO. Our goal is to establish a plausible reaction mechanism for the dimerization.

## Experimental

**Materials.** AO (3,6-bis(dimethylamino)acridinium chloride) and $D_2O$ (99.8 atom% in deuterium) were purchased from Merck. The 10-alkyl derivatives of AO (methyl-AO, ethyl-AO, propyl-AO, pentyl-AO, and octyl-AO), shown in Fig. 1, were synthesized according to the method of Miethke and Zanker.[16] All dyes were purified by column chromatography on alumina, using methanol as an eluent; no trace of impurity was detected by the method of thin-layer chromatography for each dye. All other chemicals used were reagent grade and water was glass-redistilled. Spectrophotometric and kinetic experiments were conducted in 0.1 M† NaCl and 5 mM phosphate buffer solution (ionic strength $I$=0.11; pH=6.9).

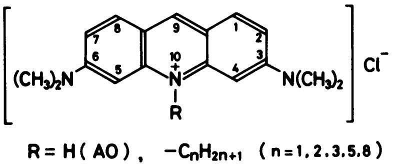

Fig. 1. Structural formulas of AO and its 10-alkyl derivatives.

**Method.** Spectrophotometric measurements were carried out with a Shimadzu UV-200S spectrophotometer. $^1$H NMR spectra of AO and methyl-AO were recorded using a 200-MHz JOEL FX-200 Fourier transform spectrometer. The measurements were performed in the concentration range $10^{-2}$ M to $5\times10^{-4}$ M. The sample solutions were prepared in $D_2O$ so as to contain a small amount of internal reference sodium 2,2-dimethyl-2-silapentane-5-sulfonate(DSS). Kinetic experiments were performed with a Unisoku TF-102 fluorescence temperature-jump spectrophotometer. This equipment is one of joule-heating type. The light source was a 250 W halogen lamp and the path length of the cell was 5 mm. The time cource of temperature rise could be approximated by an exponential function and its time constant ($\tau_r$) was in the range of 4—5 μs, depending on temperature and concentration of salt. Data were obtained at the temperature rise of 5 °C. Since the relaxation times ($\tau$) for the present systems were close to $\tau_r$, especially at high temperatures and high concentrations of dye, they were determined by the deconvolution method.[17] The relaxation curve observed by an imposition of an exponential type perturbation can be expressed by

$$X(t) = X_0\left\{\frac{\tau}{\tau-\tau_r}\exp(-t/\tau)-\frac{\tau_r}{\tau-\tau_r}\exp(-t/\tau_r)\right\} \qquad (1)$$

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† 1 M=1 mol dm$^{-3}$.

where $X$ and $X_0$ are an observable and its relaxation amplitude, respectively. $\tau$ was evaluated using a nonlinear regression analysis. All the calculations were carried out on an NEC ACOS-850 computer system.

## Results

**Equilibrium constants and Thermodynamic Parameters of Dimerization.** Figure 2 shows typical absorption spectra observed for methyl-AO in 0.1 M NaCl solution. Similar spectral changes were also obtained with AO and other 10-alkyl derivatives. The presence of clear isosbestic points (475 and 518 nm) in the absorption spectra indicates an equilibrium between two spectral species, monomer and dimer (Fig. 2). Hence, the data were analyzed on the basis of monomer-dimer equilibrium.

$$2\mathrm{D} \overset{K_\mathrm{D}}{\rightleftharpoons} \mathrm{D}_2 \tag{2}$$

The apparent molar extinction coefficient ($\varepsilon$) at a given wavelength can be expressed by

$$\varepsilon = \varepsilon_\mathrm{M}\alpha + \varepsilon_\mathrm{D}(1-\alpha) = (\varepsilon_\mathrm{M}\text{-}\varepsilon_\mathrm{D})\alpha + \varepsilon_\mathrm{D}, \tag{3}$$

where $\varepsilon_\mathrm{M}$ and $\varepsilon_\mathrm{D}$ are the molar extinction coefficients per monomer unit in the monomer and dimer forms, respectively, and $\alpha$ is the fraction of dye present in the monomer form. $\alpha$ can be estimated from the dimerization constant $K_\mathrm{D}$ and the total concentration of dye $C_\mathrm{T}$ as

$$\alpha = \frac{(1+8K_\mathrm{D}C_\mathrm{T})^{1/2}-1}{4K_\mathrm{D}C_\mathrm{T}}. \tag{4}$$

The value of $K_\mathrm{D}$ was determined by the reiteration method. First an arbitrary $K_\mathrm{D}$ is chosen and then the corresponding values of $\alpha$ for several concentrations are calculated using Eq. 4. For each wavelength, the best straight-line fit of $\varepsilon$ vs. $\alpha$ is found by linear least-squares method. The procedure is repeated to self-consistency. Dimer and monomer spectra were obtained from the intercepts of the straight lines at $\alpha$=0 and $\alpha$=1. Assuming that the planes of two component molecules in a dimer are parallel to each other, intermolecular distance $R$ was estimated according to the method of Monahan and Blossey.[18] From temperature dependence of $K_\mathrm{D}$, standard enthalpy and entropy changes of dimerization were also determined. These results are summarized in Table 1.

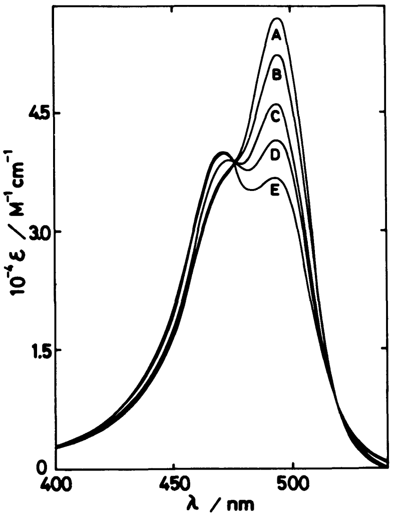

Fig. 2. Absorption spectra of methyl-AO in 0.1 M NaCl and 5 mM phosphate buffer solution ($I$= 0.11; pH=6.9) at 25 °C.
A: $4.1\times10^{-6}$ M, B: $7.2\times10^{-6}$ M, C: $1.4\times10^{-5}$ M, D: $2.38\times10^{-5}$ M, E: $3.6\times10^{-5}$ M.

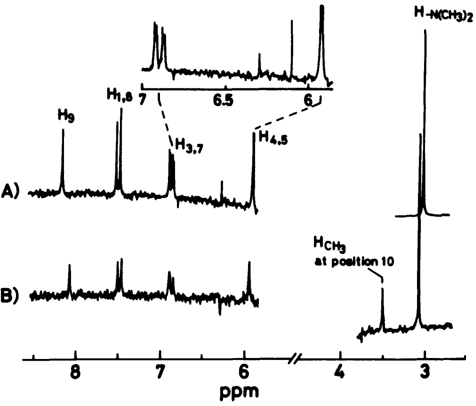

Fig. 3. $^1$H NMR spectra of (A) AO ($5\times10^{-4}$ M) and (B) methyl-AO ($5\times10^{-4}$ M) in $D_2O$.

Table 1. Equilibrium and Thermodynamic Parameters for Dimerization of AO and Its 10-$N$-alkyl Derivatives at 25 °C, $I$=0.11

| | $10^{-4}K_\mathrm{D}$ / M$^{-1}$ | $-\Delta H^\circ$ / kJ mol$^{-1}$ | $-\Delta S^\circ$ / J K$^{-1}$ mol$^{-1}$ | $R$ / Å |
|---|---|---|---|---|
| AO | 1.60 (1.0[a], 1.08[c]) | 56.9 (37.4[a], 28.7[c]) | 111 (41.3[a], 19.0[c]) | 6.1 |
| Methyl-AO | 1.70 (0.71[b], 1.08[c]) | 57.0 (29.7[b], 29.0[c]) | 110 (26.0[b], 20.0[c]) | 6.4 |
| Propyl-AO | 2.65 (0.85[b], 1.65[c]) | 57.3 (29.3[b], 29.0[c]) | 108 (23.0[b], 16.5[c]) | 6.8 |
| Pentyl-AO | 3.03 (1.07[b], 2.22[c]) | 58.6 (28.6[b], 28.6[c]) | 110 (19.0[b], 12.7[c]) | 6.8 |
| Octyl-AO | 2.90 (1.89[b]) | — (24.3[b]) | — (0.0[b]) | — |

a) 1 mM phosphate buffer (pH=6.9) at 25 °C.[6,7] b) Water, at 25 °C.[13] c) 1 mM HCl, at 20 °C.[10,11] Listed values for $K_\mathrm{D}$ at 25 °C were calculated from $\Delta H^\circ$ and $\Delta S^\circ$ using the equation $K_\mathrm{D}=\exp\{-(\Delta H^\circ-T\Delta S^\circ)/RT\}$.

**Configuration of AO and Methyl-AO Dimers.** Figure 3 shows $^1$H NMR spectra of AO and methyl-AO in $D_2O$. In agreement with the assignment reported by Blears and Danyluk,[14] the NMR spectrum of AO was assigned, in order of decreasing field, to resonances due to methyl protons (3.02 ppm), 4,5-protons (5.92 ppm), 2,7-protons (6.89 ppm), 1,8-protons (7.51 ppm), and 9-proton (8.19 ppm). A clear splitting due to long-range coupling between 2,7- and 4,5-protons was also observed (Fig. 3). In the case of methyl-AO, an additional resonance due to the methyl protons at the position 10 was observed at 3.51 ppm (Fig. 3). Concentration dependence of the spectra shows similar dilution shifts to low field as observed for AO by Blears and Danyluk.[14] Table 2 lists the dilution shift of each resonance, given in terms of the difference between two concentrations $10^{-2}$ M and $5\times10^{-4}$ M.

**Kinetic Parameters.** The square of the reciprocal relaxation time for the dimerization reaction (Eq. 2) can be expressed by[19]

$$\tau^{-2} = 8k_F k_B C_T + k_B^2, \tag{5}$$

where $k_F$ and $k_B$ are the forward and backward rate constants, respectively. Figure 4 shows plots of $\tau^{-2}$ vs. $C_T$ for AO and its five 10-alkyl derivatives, indicating that the linear relationship does hold for the present system in agreement with Eq. 5. The rate constants and $K_D$ $(=k_F/k_B)$ obtained from these plots are listed in Table 3. This table also summarizes thermodynamic parameters ($\Delta H^0$ and $\Delta S^0$) determined kinetically and activation parameters determined according to Eyring's absolute rate theory. Activation enthalpy for the forward reaction $\Delta H_F^{\neq}$ has the value nearly equal to zero for all dyes ($\Delta H_F^{\neq} \sim 0 \pm 6$ kJ mol$^{-1}$), while that for the backward reaction $\Delta H_B^{\neq}$ has the value in the range 60—70 kJ mol$^{-1}$ (Table 3). The forward and backward activation entropies were found to be $\Delta S_F^{\neq} = -40$—$-80$ JK$^{-1}$ mol$^{-1}$ and $\Delta S_B^{\neq} = 60$—95 JK$^{-1}$ mol$^{-1}$, respectively (Table 3). The experimental error for the rate constants was estimated to be ±10—20%, while that for the thermodynamic parameters was ±20—30%. Considering this point, it seems likely that both kinetically and spectrophotometrically determined thermodynamic parameters are in agreement with each other.

Table 2. Observed Dilution Shifts for AO and 10-Methyl-AO, and Ring Current Shielding

| Proton | Dilution shift of AO in ppm | Dilution shift of 10-methyl-AO in ppm | Ring current shielding in ppm |
|---|---|---|---|
| $H_1'$ | 0.34 | 0.33 | 0.25 |
| $H_2$ | 0.32 | 0.29 | 0.21 |
| $H_4$ | 0.49 | 0.46 | 0.34 |
| $H_9$ | 0.42 | 0.37 | 0.31 |
| $H_{-N(CH_3)_2}$ | 0.18 | 0.18 | |
| $H_{CH_3}$[a] | | 0.41 | |

a) Methyl proton at position 10.

## Discussion

Constantino et al.[11] and Septinus et al.[13] found that the values of $K_D$, $\Delta H^0$, and $\Delta S^0$ for the dimerization of 10-alkyl derivatives of AO in water and a dilute HCl solution have a tendency to become large with an increase in the length of the alkyl chain (Table 1). They attributed this tendency to the increase of

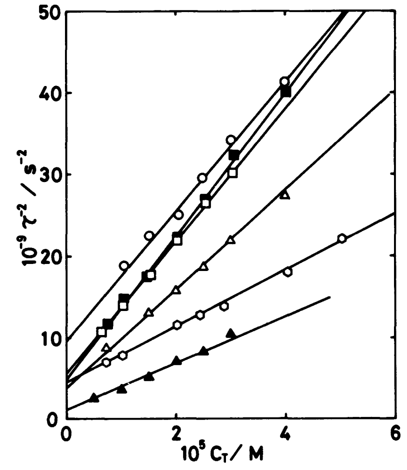

Fig. 4. Plots of $\tau^{-2}$ vs. $C_T$. ○: AO, ⬡: methyl-AO, □: ethyl-AO, ■: propyl-AO, △: pentyl-AO, ▲: octyl-AO.

Table 3. Summary of Kinetic Results

| | $10^{-4}\ K_D$ / M$^{-1}$ | $10^{-9}\ k_F$ / M$^{-1}$ s$^{-1}$ | $10^{-4}\ k_B$ / s$^{-1}$ | $-\Delta H^\circ$ / kJ mol$^{-1}$ | $\Delta H_F^{\neq}$ / kJ mol$^{-1}$ | $\Delta H_B^{\neq}$ / kJ mol$^{-1}$ | $-\Delta S^\circ$ / JK$^{-1}$ mol$^{-1}$ | $-\Delta S_F^{\neq}$ / JK$^{-1}$ mol$^{-1}$ | $\Delta S_B^{\neq}$ / JK$^{-1}$ mol$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|
| AO | 1.0 (1.8[a]) | 1.0 (1.0[a]) | 9.8 (5.6[a]) | 65 | | | 140 | | |
| Methyl-AO | 0.97 | 0.65 | 6.7 | 65 | | | 140 | | |
| Ethyl-AO | 1.8 | 1.3 | 7.5 | 57 | ≈0 | 60—70 | 110 | 40—80 | 60—95 |
| Propyl-AO | 2.3 | 1.6 | 7.0 | 59 | | | 110 | | |
| Pentyl-AO | 2.0 | 1.2 | 6.2 | 64 | | | 130 | | |
| Octyl-AO | 3.4 (2.2[b]) | 1.0 (0.77[b]) | 3.2 (3.4[b]) | — | | | — | | |

a) 1 mM phosphate buffer and 0.1 M NaCl at 23 °C and pH=6.9.[7] b) 0.05 M NaF at 23 °C.[8]

hydrophobic interactions, that is, to the stabilization of water structure surrounding the introduced alkyl group. Septinus et al.[13] also pointed out that the longer the alkyl chain becomes, the more the tendency is pronounced. The values of $\Delta H^0$ and $\Delta S^0$ in 0.1 M NaCl solution (Table 1) exhibit no clear dependence on the length of the alkyl chain, whereas the $K_D$ value clearly shows that the stabilization of dimer due to the introduction of alkyl chain takes place as in the case of water and a dilute HCl solution. Furthermore, the comparison of the present results with those in water and a dilute HCl solution shows that the presence of salt leads to the increase of $K_D$. This can be interpreted in terms of the stabilization of dimer by the electrostatic screening of positive charges.[6] Table 1 also shows that NaCl has a significant effect upon $\Delta H^0$ and $\Delta S^0$. The change in $\Delta H^0$ by the presence of NaCl is compensated by the corresponding change in $\Delta S^0$ to minimize the change in $\Delta G^0$. Such enthalpy–entropy compensation has scarecely been reported for the dimerization of dye molecules. By comparison of two reports concerning the dimerization of proflavine,[20,21] however, one can find the same behavior; $\Delta H^0 = -23$ kJ mol$^{-1}$ and $\Delta S^0 = -25$ JK$^{-1}$ mol$^{-1}$ in 0.05 M acetate buffer solution[20] and $\Delta H^0 = -60$ kJ-mol$^{-1}$ and $\Delta S^0 = -150$ JK$^{-1}$ mol$^{-1}$ in 0.2 M KCl solution.[21] The marked decrease in $\Delta H^0$ and $\Delta S^0$ upon addition of salt suggests an alteration in the hydration structure surrounding dye molecules. In this connection, it should be noted that Cl$^-$, which forms a negative charge cloud around dye molecules, is well-known as an order-destroying ion of water structure.[22] The effect of Cl$^-$ upon water structure may be responsible for the change in the thermodynamic parameters. The origin of this effect is at present unclear but is being explored in our laboratory.

As can be seen in Table 2, the dilution shifts of proton resonances of AO and methyl-AO show almost the same behavior, suggesting the same dimer configuration. Using the theory of Johnson and Bovey[23] and assuming that the dye molecules are arranged in parallel so that the centers of adjacent acridine rings are exactly superimposed or they are laterally displaced,[14] the shielding due to the ring currents of adjacent rings was estimated as a function of the interplanar distance ($R$) and the distance by which adjacent rings are off-center with respect to each other (B). The relative shifts[14] of proton resonance were found to agree well with the ring current schieldings calculated for the configuration in which ring nitrogens of adjacent dye molecules are in opposite direction and $R=6$ Å and $B=0.5$ Å (Table 2). This interplanar distance (6 Å) coincides with the values obtained from the spectrophotometric analysis (Table 1). Furthermore, the magnitude of the dilution shifts for 10-methyl protons is several times larger than that for dimethylamino protons, showing that the 10-methyl group is placed at a position where the protons are readily exposed to the field due to the ring currents of adjacent rings. Such a position can be attained by the lateral displacement between the centers of adjacent rings; this is in harmony with the above dimer configuration.

Table 3 shows that the association and dissociation rate constants vary, in a complex manner, with increasing length of the introduced alkyl chain. To understand this behavior in more detail, it is necessary to examine the following general mechanism for the dimer formation.

$$\mathrm{D} + \mathrm{D} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{D,\,D} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{D_2}. \tag{6}$$

The first step is the diffusion-controlled formation of an outer-sphere complex. The second step is the formation of a stable dimer accompanying a rearrangement of dye monomers and ejection of water molecules. Applying the steady-state assumption to the outer-sphere complex, the observed rate constant can be expressed by

$$\begin{aligned} k_F &= \frac{k_1k_2}{k_{-1}+k_2}, \\ k_B &= \frac{k_{-1}k_{-2}}{k_{-1}+k_2}. \end{aligned} \tag{7}$$

The diffusion controlled-rate constants $k_1$ and $k_{-1}$ for AO can be theoretically calculated[24] to be $k_1=4.5\times10^9$ M$^{-1}$ s$^{-1}$ and $k_{-1}=5.3\times10^9$ s$^{-1}$, assuming that the diffusion coefficient of AO is $5\times10^{-10}$ m$^2$ s$^{-1}$[7] and the encounter distance is 9 Å; the encounter distance was

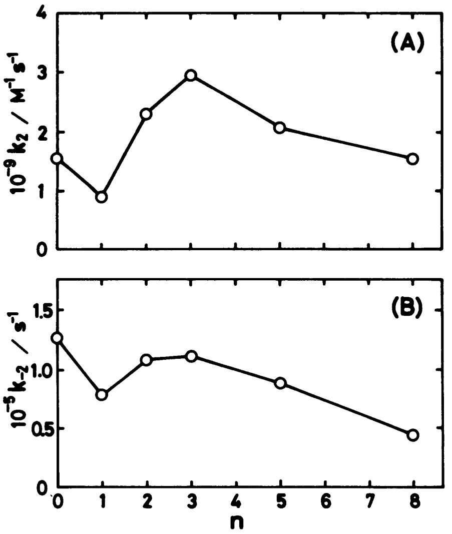

Fig. 5. The dependence of the rate constants associated with the second step in Eq. 8 on the length of the introduced alkyl chain. Abscissa shows the number of carbon atoms in the alkyl chain. (A): forward rate constant, (B): backward rate constant.

estimated as the sum of the interplanar distance in dimer (6 Å) and the diameter of water molecule (2.8 Å). Substituting these values and the values of $k_F$ and $k_B$ (Table 3) into Eq. 7, the rate constants associated with the second step can be determined. The rate constants for other dyes can also be estimated in the same manner by using the diffusion coefficients evaluated with the Stokes-Einstein relation for a spherical model. The evaluation of the diffusion coefficients may not be strictly valid. However, the uncertainty is unlikely to affect the conclusions of the present paper. Figure 5 indicates that both $k_2$ and $k_{-2}$ vary with the length of the alkyl chain in a similar manner as $k_F$ and $k_B$ (Table 3). In the case of methyl-AO, the relatively slow rates can be ascribed either to a high-energy activated state, or to the stabilization of the outer-sphere complex and the dimer, or to both. The dimer configuration determined from the $^1$H NMR spectra suggests that an alteration in the hydration structure rather than a direct interaction between the introduced methyl groups plays a significant role. The rate constants for ethyl- and propyl-AO have somewhat larger values, suggesting that the alkyl chains containing two or three carbon atoms make the activated configuration be easily accessible. For AO-derivatives having more longer alkyl chains, the rate decrease with an increase in the chain length. This effect may be attributed to the stabilization of both the outer-sphere complex and the dimer by direct hydrophobic interactions between hydrocarbon chains.[13] Dewey et al.[25,26] have shown that specific solvent-solute interactions play an important role in the kinetics of the dimerization of proflavine and thionine in aqueous alcohol solutions. In the case of methyl-AO, such interactions may be responsible for the relatively slow rates.

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