Salt Effects on Equilibrium and Kinetics of the Reaction of Tetrakis(1-methyl-4-pyridinio)porphine with DNA

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The association reaction of tetrakis(1-methyl-4-pyridinio)porphine (TMpyP) with salmon testes DNA has been investigated spectrophotometrically, statically, and kinetically in buffers containing different concentrations of Na^+. Free TMpyP had an absorption maximum at 424 nm, which decreased with increasing DNA concentration. At the low Na^+ concentrations of less than 1.20×10^{-1} mol dm^{-3} the minimal absorbance at the wavelength was reached by less than 1×10^{-4} mol dm^{-3} DNA. On the other hand, near the end of the titration at the high DNA concentrations at 2.20×10^{-1} mol dm^{-3} Na^+, the absorbance no longer continued to decrease but displayed a small absorbance increase. The dependence of the association constant on the Na^+ concentration indicated that less than one ion-pair contributed to a formation of the complex between TMpyP and DNA if there was the same reaction mechanism at the Na^+ concentration range used in the experiment. These results of the spectra and the association constants suggested that a mechanism of the reaction might be different at the low and high salt concentrations. Kinetic results by a micro stopped-flow method showed that the reaction was a single step at the low Na^+ concentration, while at the high Na^+ concentration it consisted of two steps.

In many researches of drugs binding to nucleic acids, well studied were the interactions of DNA with ethidium and acridine which were able to slip between adjacent base pairs of double-stranded DNA without requiring distortions of DNA since the drugs were small, planar, and aromatic.[1] Recent investigation of drug-nucleic acid interactions has led to the unanticipated result that the association reaction of propidium to poly(A)·poly(U) plays different behaviors at the low and high salt concentrations.[2] That is, the reaction of propidium, a dication having a large and charged sidechain, binding to poly(A)·poly(U) is a single step at the low Na^+ concentration, while at the high Na^+ concentration it consists of two steps which contain the slow unimolecular process after the binding step.

A nonmetal and metal derivative of tetraphenylporphine may be expected to provide severe steric hindrance for intercalation since its phenyl meso-substituents are perpendicular to the plane of the porphyrin ring.[3] In fact, the excellent works of Pasternack et al.[4,5] and Wilson et al.[6] have indicated that the derivative of some metals such as Zn, Co, Fe, and Mn hardly interacts with poly(dG-dC)$_2$, and nonmetal tetraphenylporphine in the reaction with poly(dG-dC)$_2$ and poly(dA-dT)$_2$ prefers to bind to GC sites at low ionic strength and to AT site at high ionic strength.

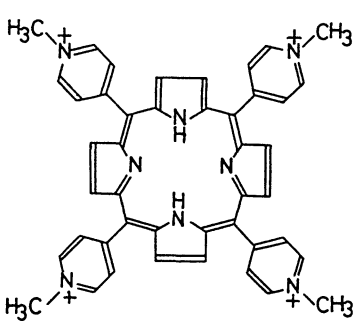

Fig. 1. The structure of tetrakis(1-methyl-4-pyridinio)porphine (TMpyP).

How does a nonmetalloporphyrin interact with the other heterogeneous nucleic acid? How does a salt affect a reaction of the drug with the nucleic acid? In this paper, we report salt effects on equilibrium and kinetics of the reaction of tetrakis(1-methyl-4-pyridinio)porphine (TMpyP) with salmon testes DNA. TMpyP is a large tetracation as shown in Fig. 1.

Experimental

Materials. Salmon testes DNA and 5,10,15,20-tetrakis(1-methyl-4-pyridinio)porphine (TMpyP) were obtained from Sigma Chemical Co. and Dojin Chemical Co., respectively. All solutions were prepared using a buffer consisting of 10^{-2} mol dm^{-3} Na_2HPO_4 and 10^{-4}mol dm^{-3} Na_2EDTA, pH 7.0. Each buffer solution was adjusted to the desired final Na^+ concentration by adding NaCl and followed by readjustment of the pH to 7.9. The Na^+ concentration was calculated as the sum of the concentrations from NaCl, Na_2HPO_4 and Na_2EDTA. The solutions of DNA were prepared and dialyzed against each buffer containing the desired Na^+ concentration at 4 °C for one day. The DNA and TMpyP concentrations were determined spectrophotometrically using the following extinction coefficients; $\varepsilon_{260}=6.55\times10^3$ mol^{-1} dm^3 cm^{-1} for DNA[7] and for $\varepsilon_{424}=2.26\times10^5$ mol^{-1} dm^3 cm^{-1} for TMpyP.[4]

Apparatus. Absorbance measurements in the UV-visible region were made on a Hitachi U-3200 programable spectrophotometer. Wavelength scans were made in cells having a path length of 10 mm. Cell holders were thermostated by a Hitachi SPR-7 temperature controller. Kinetic measurements were performed on an Otuka Denshi RA-401 stopped-flow apparatus and a micro stopped-flow one (Wakenyaku Co.) interfaced to a NEC PC9801-VX computer. Only 1.7×10^{-5} dm^3 of each sample solution was needed to measure each kinetic run on the micro stopped-flow apparatus. In all experiments, temperature was kept at 25.0±0.1 °C.

Equilibrium Measurements. The absorbace of TMpyP at 424 nm obeyed the Beer-Lambert law at the concentration range used in the experiments (10^{-6}—10^{-5} mol dm^{-3}). The

absorbance change of TMpyP with increasing DNA concentration at the wavelength was measured in each buffer to make a spectrophotometric titration. Following each addition of DNA, a time scan was made on the absorbance at the wavelength to confirm that the equilibrium was reached, and then the absorbance was recorded. In all experiments temperature was kept at 25.0±0.1 °C. The value of r which was defined as the average fraction of lattice binding sites of the nucleic acid occupied by the drug was calculated from the titration curve, finally being obtained an association constant of TMpyP with DNA in each solution.

Kinetic Measurements. The rates of the association of TMpyP with DNA at 3.02×10^{-2} and 2.20×10^{-1} mol dm^{-3} Na^+ were determined by monitoring the change of absorbance with time at 424 nm. In the experiments the initial concentration of DNA was always regulated to large excess over that of TMpyP. The obtained kinetic runs were analyzed with nonlinear least-squares fitting to single or double exponential curve.

Results and Discussion

Spectra of TMpyP with DNA. Free TMpyP had the Soret maximum at 424 nm, and the reaction with DNA led to a substantial red shift of the maximum and a large hypochromicity as shown in Fig. 2. The spectral shapes in Fig. 2 were very similar in each solution containing a different Na^+ concentration of less than 1.20×10^{-1} mol dm^{-3}. The reaction of TMpyP with poly(dG-dC)$_2$ led to a very large hypochromicity at the maximum and a new maximum at 444 nm, while an absorbance change with poly(dA-dT)$_2$ displayed a small red shift and hypochromicity.[4] The absorbance change and shift in the Soret region for TMpyP with DNA at the low salt concentrations are similar to those for the drug with poly(dG-dC)$_2$. However, the change of the spectrum for TMpyP with DNA at 2.20×10^{-1} mol dm^{-3} Na^+ were intermediate between those for poly(dG-dC)$_2$ and poly(dA-dT)$_2$.[4] Therefore, at the high salt concentration there appear to be interactions between TMpyP and salmon testes DNA at both GC and AT sites of the nucleic acid.

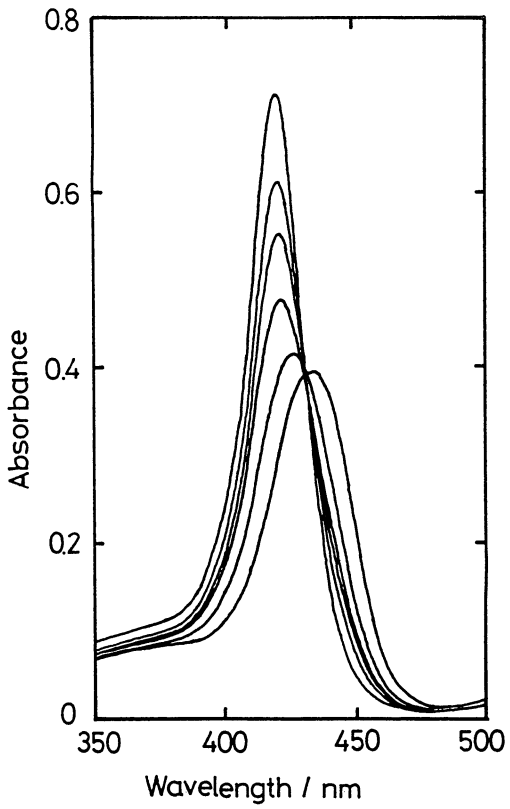

Fig. 2. Spectrophotometric titration of TMpyP with DNA at 2.02×10^{-2} mol dm^{-3} Na^+, pH 7.0 at 25 °C. The constant concentration of TMpyP is 2.87×10^{-6} mol dm^{-3}. The concentration range of DNA is from zero at the top curve to 4.67×10^{-5} mol dm^{-3} at the bottom curve at 424 nm.

The absorbance of TMpyP at 424 nm decreased with increasing DNA concentration and the minimal absorbance at the wavelength was reached by less than 1×10^{-4} mol dm^{-3} DNA at the low Na^+ concentrations of less than 1.20×10^{-1} mol dm^{-3}. However, near the end of the titration at high DNA concentrations at 2.20×10^{-1} mol dm^{-3} Na^+, the absorbance no longer continued to decrease but displayed a small absorbance increase. The behavior of the absorbance increase had been also found for the poly(dA-dT)$_2$ titration.[4] These results suggest that a large amount of Na^+ may make TMpyP to favor the AT sites of the heterogeneous DNA.

Association Constants. The results of the titration were converted to binding isotherms. The data were analyzed with the theory for site exclusion binding:[4,8]

$$\lim_{r\to0}[d(r/b)/dr]=-K(2n-1), \quad (1)$$

where b is the molar concentration of a free TMpyP, K the association constant of TMpyP with DNA, and n the number of consecutive lattice residues made inac-

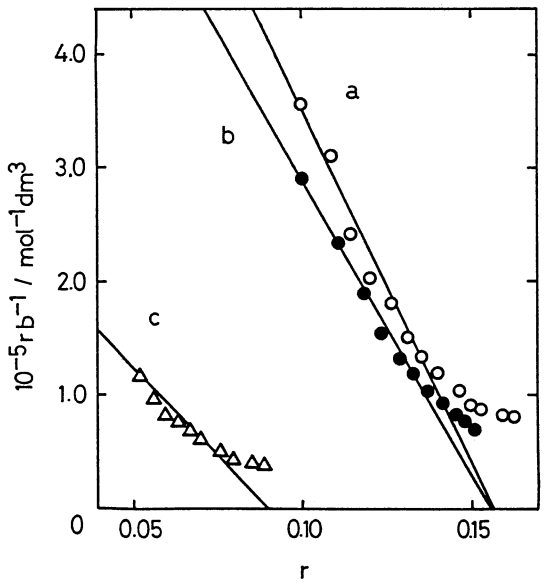

Fig. 3. Binding isotherms of TMpyP to DNA at (a) 7.02×10^{-2}, (b) 1.20×10^{-1}, and (c) 2.20×10^{-1} mol dm^{-3} Na^+ at 25 °C.

cessible by the binding of a single TMpyP molecule. Typical plots at 7.02×10^{-2}, 1.20×10^{-1}, and 2.20×10^{-1} mol dm^{-3} Na^+ are shown in Fig. 3. The obtained values of K and n are listed against the Na^+ concentrations in Table 1.

The value of K at 2.02×10^{-2} mol dm^{-3} Na^+ was 1.14×10^6 mol^{-1} dm^3. This value is smaller than 8.9×10^6 and 1.3×10^8 mol^{-1} dm^3 for ethidium and propidium, respectively, binding to salmon testes DNA at 1.6×10^{-2} mol dm^{-3} Na^+,[7] though TMpyP has more positive charges than ethidium and propidium. The result suggests that all four positive charges of TMpyP may not contribute to the binding with DNA.

Salt Effect on Association Constants. The binding constant of TMpyP with DNA decreased with increasing the Na^+ concentration. In other words, the binding is not only stacking but electrostatic in origin.[9] The simple polyelectrolyte theory[10,11] is often used in order to interpret the effects of counterion concentration on ligand binding to polyelectrolytes. Application of this theory to the interaction of a nucleic acid with a small molecule shows the relation of K and Na^+ concentration, $[Na^+]$, in Eq. 2:

$$(\partial \log K/\partial \log[Na^+]) = -m'\psi, \quad (2)$$

where $\psi = \psi_C + \psi_S$,

$\psi_C = 1 - \xi^{-1}$,

$\psi_S = (2\xi)^{-1}$,

and $\xi = q^2/\varepsilon k_B T h$.

Here, q is the elementary charge, ε the bulk dielectric constant, k_B Boltzmann's constant, T absolute temperature, h the average distance between charges on the backbone of the nucleic acid, ψ the sum of the fraction of counterions which are directly condensed onto the nucleic acid (ψ_C) and the fraction of screening counterions per phosphate (ψ_S), and m' the number of ion pairs that form with DNA on binding of one ligand.

Figure 4 shows the plots of log K vs. $-\log[Na^+]$ for TMpyP binding to DNA. The slope of the linear line in Fig. 4 was 0.49. When the average axial charge spacing along the helical axis for the DNA is assumed to be 0.17 nm,[12] the value of ψ is calculated to be 0.88 at 25 °C. Therefore, the value of m' was 0.56, surprisingly suggesting that less than one ion-pair contributes to a formation of the complex between TMpyP and DNA if there is the same reaction mechanism at the Na^+ concentration range used in the experiment.

Table 1. The Dependence of K and n for TMpyP Binding to DNA on Na^+ Concentration at 25 °C[a]

$10^2[Na^+]$/mol dm^{-3}	$10^{-5}K$/mol^{-1} dm^3	n
2.02	11.4	3.1
3.02	10.2	3.4
7.02	9.87	3.7
12.0	8.37	3.7
22.0	2.73	5.9

a) Uncertainty is within ±10% for K and n.

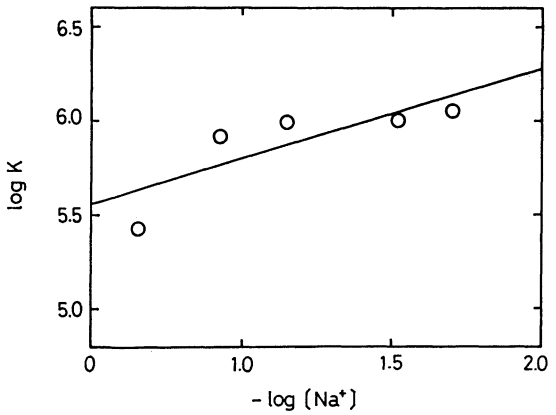

Fig. 4. The log-log plot of the association constants of TMpyP with DNA at 25 °C vs. Na^+ concentration.

It was known in the many cases of drugs binding to nucleic acids that one ion-pair contributed to a complex formation, especially in the cases of synthetic polymers, for example, ethidium-polyd(AT)·polyd(AT),[7] and propidium-poly(A)·poly(U).[13] In the case of TMpyP, Wilson et al. reported that $m'\psi$ values of TMpyP binding to poly(dG-dC)$_2$ and poly(dA-dT)$_2$ were the same, ca. 2.7.[8] Pasternack et al. also reported a $m'\psi$ value of 1.6 for TMpyP with poly(dG-dC)$_2$.[14] We obtained the value of 1.9 for TMpyP with poly(A)·poly(U).[15] The very small value of $m'\psi$ for the reaction of TMpyP with salmon testes DNA may be due to the different reaction mechanism at the low and high salt concentrations and/or the different behavior of the binding at the GC and AT sites of DNA.

Kinetics at Low Na^+ Concentration. The results of the spectra and the association constants indicate that a mechanism of the reaction between TMpyP and DNA might be different at the low and high salt concentrations. Therefore, reaction kinetics of TMpyP to DNA at 3.02×10^{-2} and 2.20×10^{-1} mol dm^{-3} Na^+ have been investigated to provide insights into salt effect on the dynamics of the binding behaviors. Figure 5 shows typical kinetic runs at 3.02×10^{-2} and 2.20×10^{-1} mol dm^{-3} Na^+. The kinetic run at the low Na^+ concentration was able to be fitted with a single exponential function. However, the run at the high Na^+ concentration was not, as shown in Fig. 5. On the kinetic curve at the high Na^+ concentration, a fast step finished within about 20 ms and was followed by the slower step.

In kinetic measurements at 3.02×10^{-2} mol dm^{-3} Na^+ the stopped-flow traces obeyed a first-order kinetic equation when the DNA concentration was in large excess over the TMpyP concentration. The plot of the observed rate constant, k_{obs}, against the initial concentration of DNA, $[D]_0$, at 25 °C was linear in agreement with Eq. 3 as shown in Fig. 6:

$$k_{obs} = k_1[D]_0 + k_{-1}. \quad (3)$$

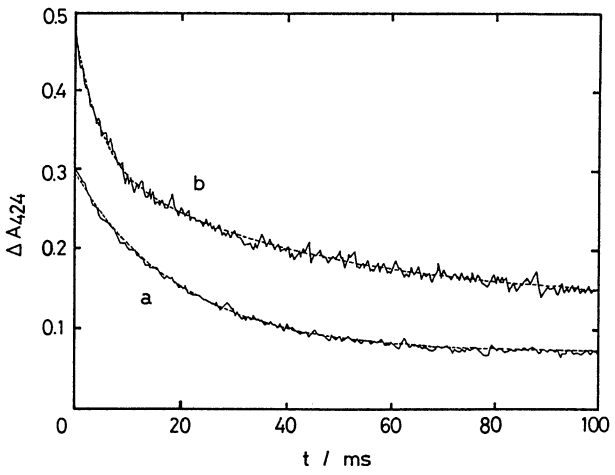

Fig. 5. Stopped-flow kinetic traces for the reaction of TMpyP with DNA at 25 °C at (a) 3.02×10^{-2} and (b) 2.20×10^{-1} mol dm^{-3} Na^+. The TMpyP concentration kept 2.8×10^{-6} mol dm^{-3} in both buffers was about 10 times smaller than DNA concentration. The smooth dotted lines represent nonlinear least-squares fitting to single and double exponential curves for curves a and b, respectively.

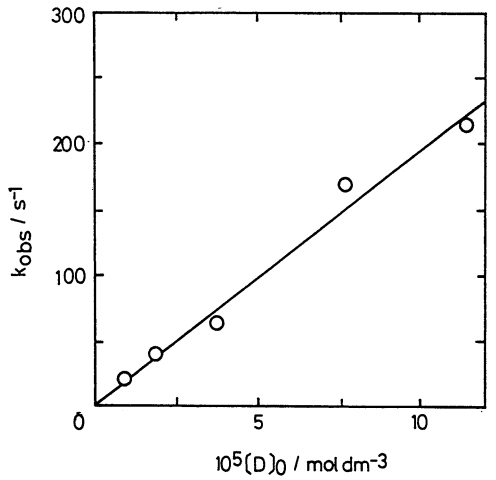

Fig. 6. Dependence of k_{obs} on DNA concentration at 3.02×10^{-2} mol dm^{-3} Na^+ at 25 °C.

In Eq. 3, k_1 and k_{-1} were the association and dissociation rate constants, respectively. The linear relation between k_{obs} and $[D]_0$ indicates the reaction path at the low Na^+ concentration is simple as described below:

$$\text{TMpyP} + \text{DNA} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{TMpyP-DNA Complex.} \quad (4)$$

The values of k_1 and k_{-1} determined from the dependence of k_{obs} on $[D]_0$ by a least-squares method were 1.93×10^6 mol^{-1} dm^3 s^{-1} and 1.9 s^{-1}, respectively. The association constant obtained by the ratio of the rate constants, k_1/k_{-1}, is 1.02×10^6 mol^{-1} dm^3 and the value is well in accord with the association constant of 1.04×10^6 mol^{-1} dm^3 by the direct determination (Table 1), which strengthens the reliability of the simple reaction mechanism at the low salt concentration described above.

Kinetics at High Na^+ Concentration. The association reaction at 2.20×10^{-1} mol dm^{-3} Na^+ gives the two observed rate constants of $k_{obs,1}$ and $k_{obs,2}$. Figure 7 shows the dependences of $k_{obs,1}$ and $k_{obs,2}$ on $[D]_0$ at 25 °C. The linear dependence of $k_{obs,1}$ and the lack of dependence of $k_{obs,2}$ on $[D]_0$ at the high Na^+ concentration cannot be explained by the simple one-step mechanism at 3.02×10^{-2} mol dm^{-3} Na^+. It could be considered that there is an allosteric conversion from some nonstandard DNA state to a usual B-form double helix before the binding of TMpyP.[16] However, the allosteric conversion cannot give the probable explanation for the dependences of $k_{obs,1}$ and $k_{obs,2}$ on $[D]_0$.[17]

The results of the spectra and the association constant at the high Na^+ concentration may suggest two most reasonable explanations for the kinetic results: 1) There is a rearrangement of TMpyP among the sites of DNA after the first binding process (Scheme 1), or 2)

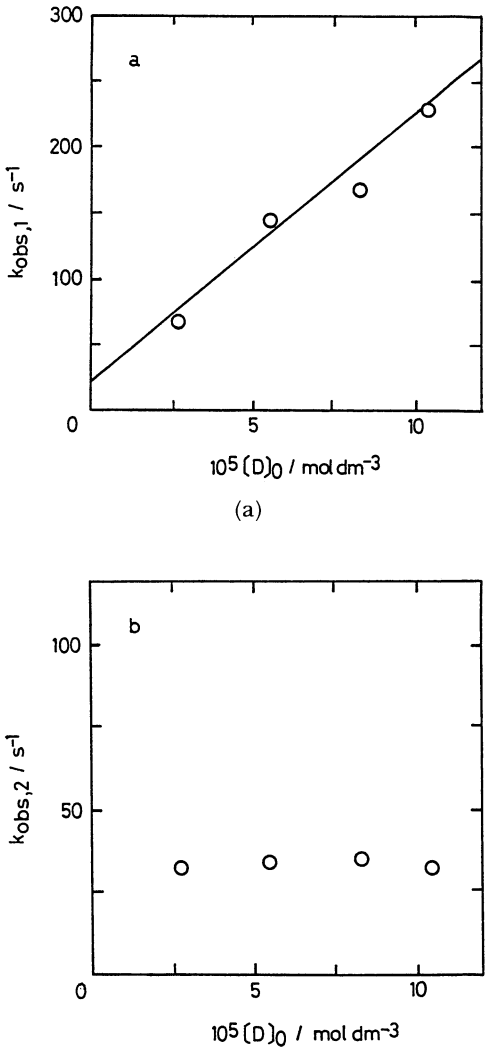

Fig. 7. Dependences of (a) $k_{obs,1}$ and (b) $k_{obs,2}$ on DNA concentration at 2.20×10^{-1} mol dm^{-3} Na^+ at 25 °C.

there are independent binding processes of TMpyP at the different sites of DNA (Scheme 2).

$$\text{TMpyP} + \text{DNA} \underset{k_{-1,1}}{\overset{k_{1,1}}{\rightleftarrows}} \text{Complex 1} \underset{k_{-2,1}}{\overset{k_{2,1}}{\rightleftarrows}} \text{Complex 2}$$

Scheme 1.

$$\text{TMpyP} + \text{DNA} \underset{k_{-1,2}}{\overset{k_{1,2}}{\rightleftarrows}} \text{Complex 1}$$

$$\text{TMpyP} + \text{DNA} \underset{k_{-2,2}}{\overset{k_{2,2}}{\rightleftarrows}} \text{Complex 2}$$

Scheme 2.

In Schemes 1 and 2, Complexes 1 and 2 are the different complexes of TMpyP with DNA. The rate constants, $k_{obs,1}$ and $k_{obs,2}$, for these mechanism are given by Eqs. 5 and 6 for Scheme 1, and Eqs. 7 and 8 for Scheme 2, respectively:

$$k_{obs,1} = k_{1,1}[D]_0 + k_{-1,1} \quad (5)$$

$$k_{obs,2} = k_{2,1}[D]_0/([D]_0 + 1/K_{1,1}) + k_{-2,1} = k_{2,1} + k_{-2,1} \text{ (at very large } [D]_0) \quad (6)$$

$$k_{obs,1} = k_{1,2}[D]_0 + k_{-1,2} \quad (7)$$

$$k_{obs,2} = k_{2,2}[D]_0/(K_{1,2}[D]_0 + 1) + k_{-2,2} = (k_{2,2}/K_{1,2}) + k_{-2,2} \text{ (at very large } [D]_0) \quad (8)$$

Here, the rate constants in Eqs. 5—8 are as indicated in Schemes 1 and 2, and $K_{1,1}=k_{1,1}/k_{-1,1}$, $K_{1,2}=k_{1,2}/k_{-1,2}$.

The previous study[4] reported that a truly intercalated species required GC base pairs and porphyrin moieties which had no axial groups. Thus, TMpyP interacts extensively with GC regions. A fundamentally different type of complex is formed at AT regions of nucleic acids. This latter type of interaction leads to an externally bound complex in which the porphyrin is located in groove and/or is only partially intercalated. It is also known that the complexes at GC and AT regions form in larger and shorter than 3 ms at 25 °C, respectively.[18] Therefore, Complexes 1 and 2 in the present work, especially in Scheme 2, may not correspond to the TMpyP-DNA complex at GC and AT sites, respectively, because both complexes show observable stopped-flow runs of half-lives longer than 3 ms. Wilson et al.[8] have found in the dissociation reaction of TMpyP from poly(dG-dC)$_2$ that the dissociation involves the slow reaction of an initial product to form an intermediate with ion pairs and then the intermediate dissociates rapidly to the free ligand and the polymer. It appears to be similar to the present case. However, in the more complex case of a native DNA like salmon testes DNA, different types of mechanisms including intercalations at mixed GC/AT sites may be occuring.[19-21]

On the other hand, from the similarity between the results of salmon testes DNA and calf thymus DNA,[18] it suggests that, as shown in Scheme 1, TMpyP might move from site to site without first dissociating into the solvent. The linear plot in Fig. 7a by a least-squares method gives 2.06×10^6 mol^{-1} dm^3 s^{-1} and 19.4 s^{-1} for $k_{1,1}$ and $k_{-1,1}$ at the first step in Scheme 1, respectively. The value of $k_{-1,1}$ is about 10 times larger than that of k_{-1} at the low Na^+ concentration. The result can be predicted by a model of an electrostatic interactions between the drug and the nucleic acid.[11,22] Although the additional study for the other nucleic acids will be able to give a clear discussion, there can be no question for this initial results that the association displays different behaviors at the low and high salt concentrations.

In conclusion, the results of the spectra and the association constants suggested that a mechanism of the reaction may be different at the low and high salt concentrations. Kinetic results by a micro stopped-flow method showed that the reaction was a single step at the low Na^+ concentration, while at the high Na^+ concentration it consisted of two steps, supporting the above suggestion.

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