Proton exchange kinetics in [d(ACGTATACGT)]$_2$-echinomycin and [d(ACGTTAACGT)]$_2$-echinomycin complexes

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Abstract Based on imino proton exchange catalysis, base-pair lifetimes and apparent dissociation constants are reported on the complexes formed by bisintercalation of echinomycin at the CpG steps of the d(ACGTATACGT)$_2$ and d(ACGTTAACGT)$_2$ duplexes. The lifetimes of the four central A·T base pairs between two echinomycin binding sites are much shorter than in the free duplexes. The destabilization of base pairs adjacent to the binding sites is propagated one additional base pair away from the binding site.

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Key words: Echinomycin; Nuclear magnetic resonance; DNA complex; Proton exchange

1. Introduction

Echinomycin, a cyclic octadepsipeptide containing two planar quinoxaline rings that bisintercalate into DNA, has antitumor activities and is now being used in phase II clinical trials as a chemotherapeutic agent [1]. Single crystal X-ray studies of echinomycin complex with d(CGTACG)$_2$ showed that the two central A·T base pairs next to the intercalation sites were Hoogsteen base-paired with the adenines in *syn* conformation [2,3]. Footprinting studies with DNase I and II and other DNA cleavage reagents demonstrated a sequence preference for echinomycin binding at CpG step and hyperreactivity of A·T stretches within 5–10 base pairs of the binding sites [4,5].

The hyperreactivity of DNA to diethylpyrocarbonate (DEPC) in the presence of echinomycin was interpreted by Mendel and Dervan [6] and Fox and Kentebe [7] in terms of an altered DNA conformation involving Hoogsteen base-pairing. However, McClean and co-workers [8] showed that DNA fragments in which the adenines were modified at the N7 position such that they could not Hoogsteen base-pair remained hyperreactive to cleavage by OsO_4. They proposed that this structural change was the unwinding of the DNA duplex induced by the intercalative binding of echinomycin [8]. In their nuclear magnetic resonance (NMR) studies of DNA oligomers with two echinomycin binding sites, Gilbert and Feigon [9] also reported that none of four central A·T base pairs of a d(ACGTATACGT)$_2$-echinomycin complex were Hoogsteen base-paired, and binding of the drug induced unwinding of the DNA which was propagated to the central ApT step. However, in contrast to the results obtained for d(ACGTATACGT)$_2$-complex, for d(ACGTTAACGT)$_2$-echinomycin complex no NMR evidence has been reported that the helix is unwound or A·T is Hoogsteen base-paired [10].

In the present work, we studied the base pair kinetics of the central A·T base pairs of the two DNA duplexes, d(ACGTATACGT)$_2$ and d(ACGTTAACGT)$_2$ where they were complexed with echinomycins (Fig. 1), using NMR of imino protons. The imino proton exchange rates provides a simple and efficient method for measuring the lifetime and for evaluating the dissociation constant of individual base pairs [11–15]. On the basis of imino proton exchange catalysis by ammonia, we found that the lifetimes of the four central A·T base pairs in both duplexes were much shorter when complexed with echinomycins than those in the free duplexes and this structural change, destabilization, is propagated through all four A·T base pairs between the two echinomycin intercalation sites.

2. Materials and methods

2.1. DNA sample preparation

The DNA oligomers, d(ACGTATACGT)$_2$ and d(ACGTTAACGT)$_2$, were synthesized on an Applied Biosystems 391A synthesizer using β-cyanoethyl phosphoramidite chemistry. The oligonucleotides were purified by Sephadex G-25 fine gel filtration column chromatography. The NMR samples of DNA oligomers were prepared by dissolving the DNA in 450 μl of 100 mM NaCl. The pH of free DNA solutions was readjusted to 6.8. Echinomycin was a gift from the National Cancer Institute, USA. Echinomycin was added to the DNA oligomers in the NMR tube (the mol ratio of drug and DNA duplex is 3:1). The mixture was sonicated at 35°C and slowly dried with a stream of N_2 gas until complete disappearance in the NMR spectrum of the free duplex imino proton peaks. The pH of the complex solutions was readjusted to 6.6.

2.2. NMR experiments

^{1}H-NMR experiments were performed on a Bruker DMX 600 spectrometer at Korea Basic Science Institute. The catalyst-induced exchange rates were measured using saturation–recovery experiments at 15°C [16–18]. The jump-and-return (JR) pulse sequence was used to suppress the solvent peak and gradient pulses (homospoil) were used for eliminating the spurious transverse solvent magnetization generated by the parts of the excitation sequence that precede the JR observation pulses [21]. Ammonium chloride was titrated to the NMR sample from a stock of 5 M solution to give a catalyst concentration over 0–0.4 M. The pH of the solution was measured before and after each experiment and adjusted to 6.8 after each addition. The concentration of the base at each buffer concentration is given by

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Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TTAA-complex, d(ACGTTAACGT)$_2$-echinomycin complex; TATA-complex, d(ACGTATACGT)$_2$-echinomycin complex; TTAA-duplex, d(ACGTTAACGT)$_2$ duplex; TATA-duplex, d(ACGTATACGT)$_2$ duplex

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Table 1
Base pair lifetimes and apparent dissociation constants for the free duplexes and echinomycin complexes

	Base pair lifetime, τ_0 (ms)				Apparent dissociation constant ($\alpha K \times 10^5$)			
	c-TTAA[a]	c-TATA	d-TTAA	d-TATA	c-TTAA	c-TATA	d-TTAA	d-TATA
T4	5.5	0.6	9.5	1.2	0.8	1.6	1.4	3.4
T5	1.4		3.1		1.4		2.9	
T6		0.7		1.8		1.3		2.1

[a]The abbreviations used in this table are: c-TTAA, TTAA-complex; c-TATA, TATA-complex; d-TTAA, TTAA-duplex; d-TATA, TATA-duplex.

$$[\text{base}]^{-1} = (1 + 10^{pK_a - pH}) \times [\text{total buffer}]^{-1} \quad (1)$$

where pK_a for the ammonia buffer is 9.25.

The saturation recovery curves for different values of t (delay time between end of saturation and observation pulse) were fitted to an exponential function

$$I(t) = I_0 + P \cdot \exp(-t/T_{rec}) \quad (2)$$

where T_{rec} was the recovery time. The parameters, T_{rec}, I_0, and P were fitted through a non-linear method of least squares. The recovery time, T_{10}, in the absence of added catalyst, was measured by the same method. Theoretically, the exchange time with the base catalyst, τ_{ex}, is expressed by

$$\tau_{ex}^{-1} = T_{rec}^{-1} - T_{10}^{-1}. \quad (3)$$

The imino proton exchange takes place via transient base pair opening through the added base catalyst or by the intrinsic catalysis of the cyclic nitrogen in the complementary base [12,15,19]. So, the exchange time is given by

$$\tau_{ex} = \tau_0(1 + k_{cl}/(k_{tr} + k_{acc})) \quad (4)$$

where k_{cl} is the base pair closing rate constant, k_{tr} is the transfer rate constant of the imino proton in the open state to the base catalyst and k_{acc} is the transfer rate constant by intrinsic catalysis. Since the following expression is valid:

$$\tau_0 = 1/k_{op},\ K_d = k_{cl}/k_{op},\ T_{acc} = 1/K_d \cdot k_{acc},\ k_{tr} = \alpha \cdot k_i \cdot [B] \quad (5)$$

where k_{op} is the base pair opening rate constant, K_d is the base pair dissociation constant, α is accessibility parameter ($0 < \alpha < 1$), k_i is the proton transfer rate from the isolated nucleotide to the base catalyst, and [B] is the base catalyst concentration [20]. The following equation can be obtained

$$\tau_{ex} = \tau_0 + 1/(K_d \cdot \alpha \cdot k_i \cdot [B] + 1/T_{acc}). \quad (6)$$

When the ammonia base concentration is too high, the base catalyzed step dominates and the exchange time is given by [21]

$$\tau_{ex} = \tau_0 + 1/(K_d \cdot \alpha \cdot k_i \cdot [B]). \quad (7)$$

In high limits of the base catalyst concentration, the exchange time depends linearly on the reciprocal base catalyst concentration. An extrapolation to infinite base concentration then gives the base pair lifetime, τ_0, When evaluating the apparent dissociation constants (αK_d), the value, k_i, used in this study is 2×10^8 s/M [15].

3. Results and discussion

The imino proton solvent exchange rates are sensitive indicators of local stability of DNA duplexes. When a base pair is broken, the exchange takes place via the formation of a hydrogen bond between the imino proton and exchange catalyst. In this open state, it can be shown that the rate of imino proton exchange is directly proportional to the concentration of catalyst. The effects of ammonia on the imino proton spectra of free duplexes and echinomycin complexes of $d(ACGTATACGT)_2$ and $d(ACGTTAACGT)_2$ at 25°C are shown in Fig. 2. We refer to these free duplexes and complexes as the TATA-duplex, TATA-complex, TTAA-duplex, and TTAA-complex, respectively. From saturation–recovery experiments on base pair imino protons in all investigated free DNA duplexes and complexes with echinomycins, the recovery time T_{rec} could be obtained by a non-linear least square fitting of the intensities or areas to Eq. 2. Fig. 3 shows the measured values of the exchange times τ_{ex} of T imino protons with the base catalyst as function of the reciprocal concentration of the base catalyst $[B]^{-1}$. The curves appear fairly linear and were fitted by linear regression to Eq. 7.

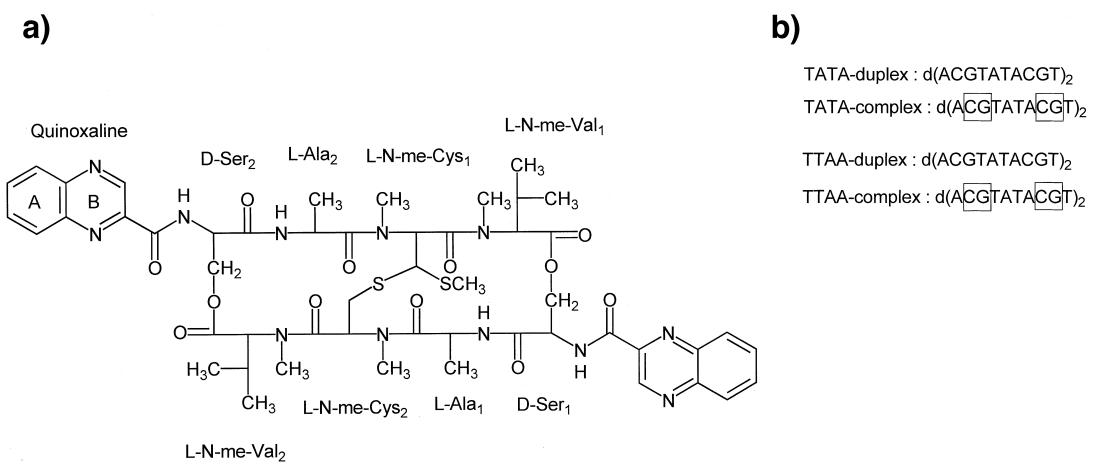

Fig. 1. a: The molecular structure of echinomycin. b: The sequences of DNA–echinomycin complexes and free DNA duplexes used in this study, where the rectangular boxes indicate the binding of echinomycin.

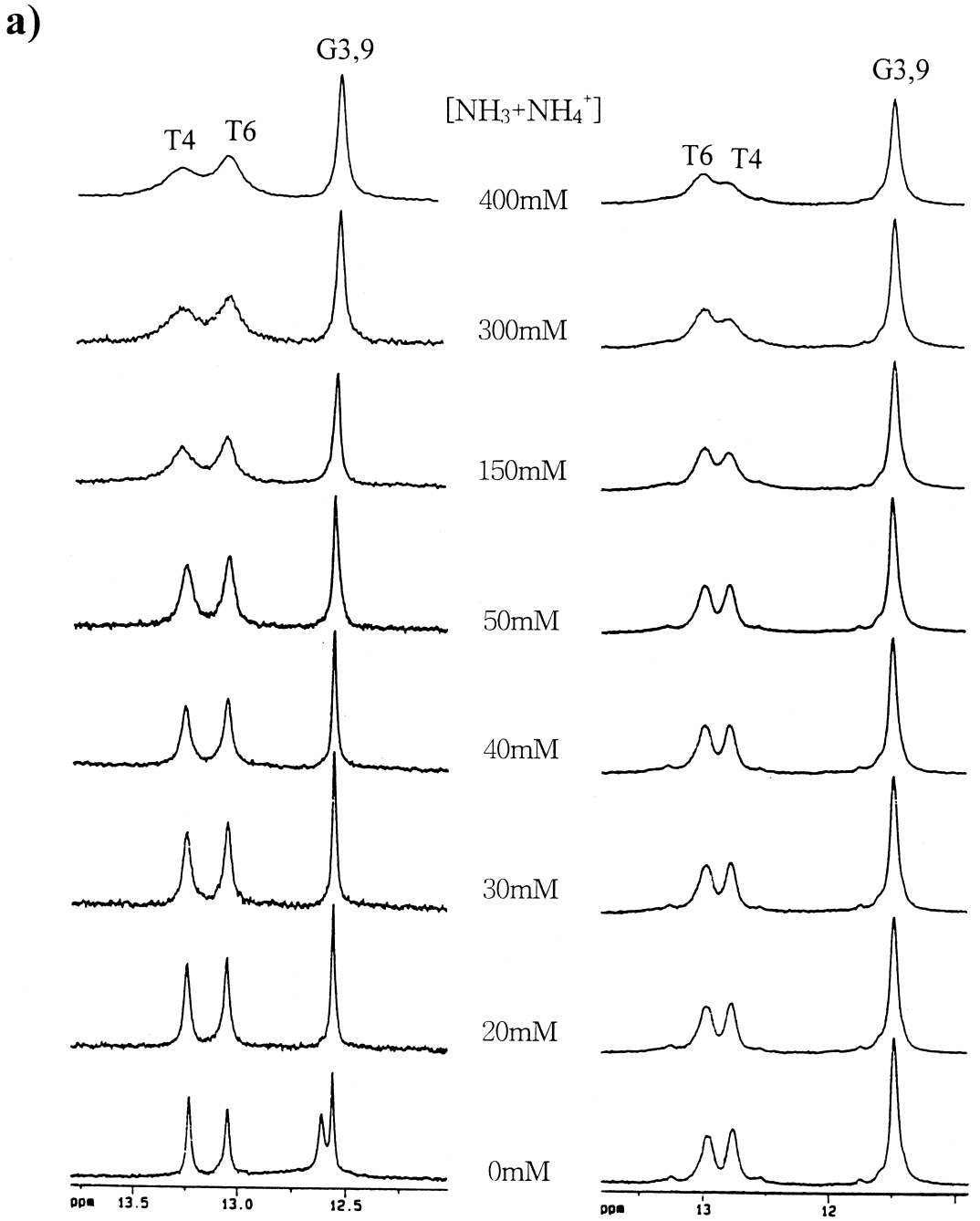

Fig. 2. a: Imino proton regions of the NMR spectra of the free DNA TATA-duplex (left) and the TATA-complex (right). b: Imino proton regions of the NMR spectra of the free DNA TTAA-duplex (left) and the TTAA-complex (right) at 15°C with varying concentrations of the exchange catalyst.

Fig. 4a shows the evaluated values of base pair lifetime τ_0 of the thymine imino protons in the DNA free duplexes and drug complexes. The result obtained for T_{10} (Fig. 4b) shows that T_{10} generally is around 200–400 ms for the free DNA duplexes and 100–200 ms for the echinomycin complexes. Two factors contribute to T_{10}, one from magnetic dipolar relaxation and the other from the exchange by intrinsic catalyst. For the TTAA-duplex the lifetime τ_0 of T4 is longer than that of T5, while T_{10} of T4 is shorter than that of T5, indicating that the exchange process by the intrinsic catalyst is less effective in T5 than in T4. The apparent dissociation constants are of the same order for the free DNA duplexes and for the complexes (Table 1). This indicates that the closing and opening rates are increased approximately by the same values as the lifetimes of the base pairs are decreased.

The base-pair lifetimes of the TTAA base pairs of the free DNA duplex are different from those of the TATA base pairs. The lifetime of the innermost A·T base pair (T5) is shorter than that of the next one (T4), while the innermost A·T base pair (T6) of TATA is longer than T4. While there is a subtle difference in the lifetimes of the two free duplexes the overall trend for the complexes is retained (Fig. 4a). Compar-

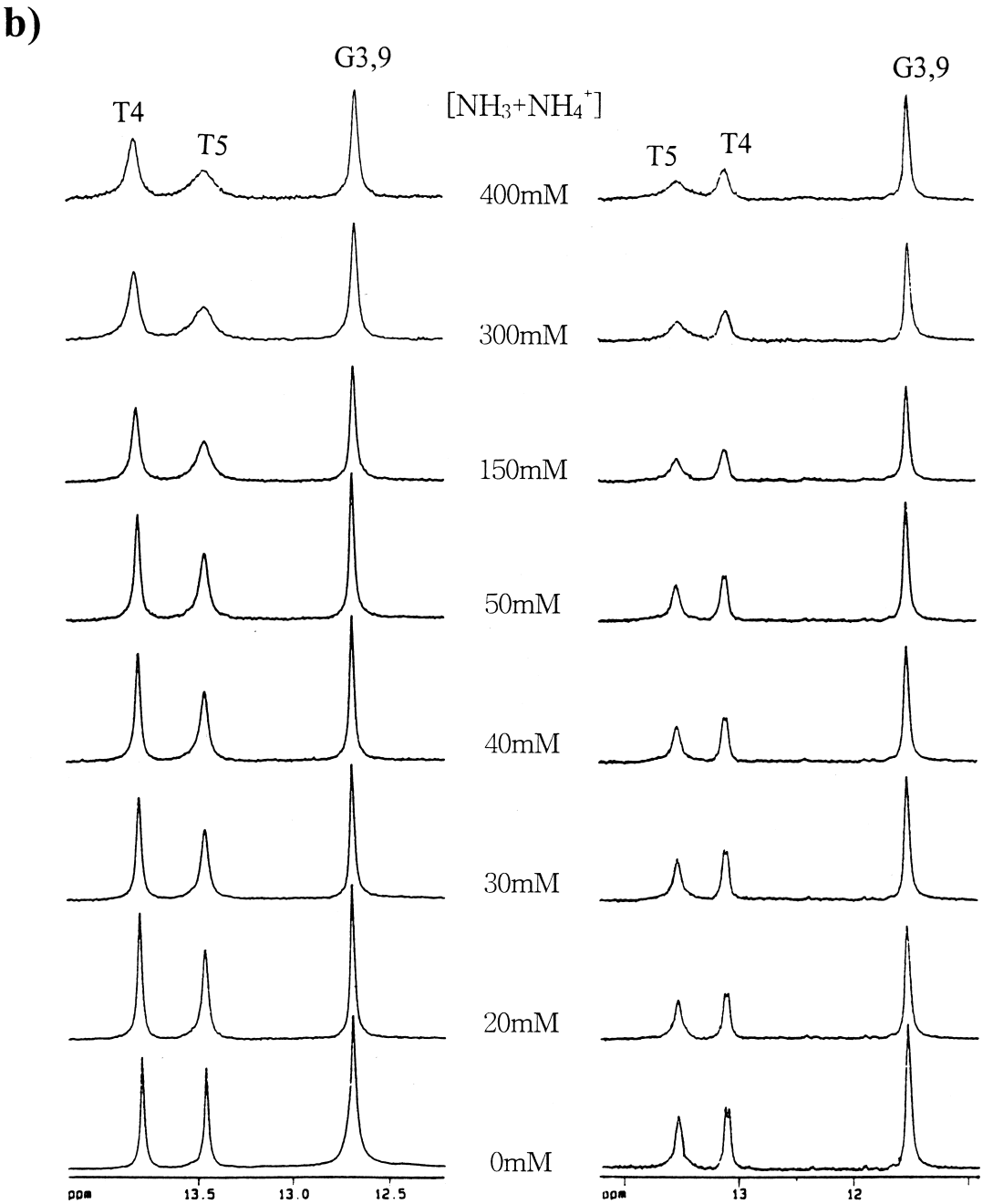

Fig. 2. (*continued*)

ison of the lifetimes of A·T base pairs in the complexes with those of the free duplexes suggests that all the A·T base pairs in the complexes are destabilized when the drugs are bound.

The increase in the exchange rate of these protons with the solvent proton indicates shorter base-pair lifetimes for the central A·T base pairs. The destabilization of the four central A·T base pairs in the TTAA-complex was confirmed by less intensive nuclear Overhauser effect (NOE) crosspeaks of T imino proton↔A–H2 than those of the free duplex (data not shown). It is noticed that the destabilization of base pairs adjacent to the binding sites is propagated one additional base pair away from the binding site. Recent results of Leroy et al. [15] also confirm this conclusion. They compared the stability of A·T base pairs adjacent to echinomycin binding sites in $d(AAACGTTT)_2$-echinomycin and $d(CCAAACGTTTGG)_2$-echinomycin, which each contained a single CG binding site. They found that these A·T base pairs were less stable in the complexes than in the free DNA. These findings are consistent with the footprinting results that have shown that the hyperreactivity of A·T rich regions adjacent to echinomycin binding sites is propagated a significant distance from the echinomycin binding site [4,5]. Gilbert and Feigon [9] suggested that unwinding of the TATA-complex was the major factor leading to hyperreactivity by the binding of echinomycin. We also observed NMR evidence for unwinding DNA duplexes in

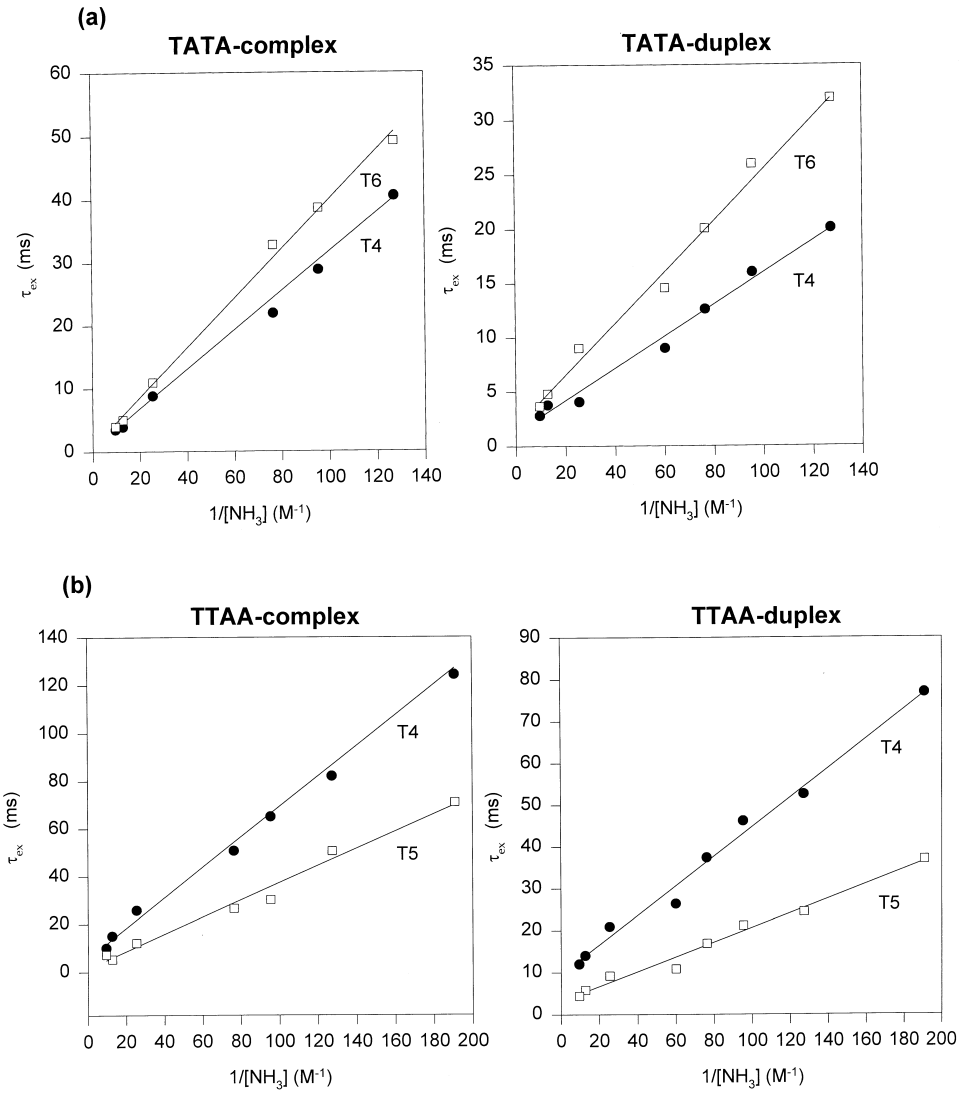

Fig. 3. a: Exchange times of T imino protons at 15°C as function of the reciprocal base catalyst concentration in the TATA-complex (left) and the free DNA TATA-duplex (right). b: Exchange times of T imino protons at 15°C as function of the reciprocal base catalyst concentration in the TTAA-complex (left) and the free DNA TTAA-duplex (right).

the TATA-complex relative to the free DNA both in the C·G base pairs between the bis-intercalated quinoxaline rings and in the central A·T base pairs [10]. However, we could not find any NMR evidence for the unwinding of the DNA duplex in the TTAA-complex [10]. No NOE was observed between C–H6 and G–H8 resonances. In addition, there was no cross-peak between T5–H6 and A6–H8 in the 50-ms mixing time NOESY spectrum. This indicates that the structure of the central region of the DNA duplex in the TTAA-complex differs from that of the TATA-complex. Thus, it appears that hyperreactivity to DNA cleavage reagents distal to echinomycin binding sites is due to two major structural changes which are destabilization and unwinding of the helix induced by drug binding.

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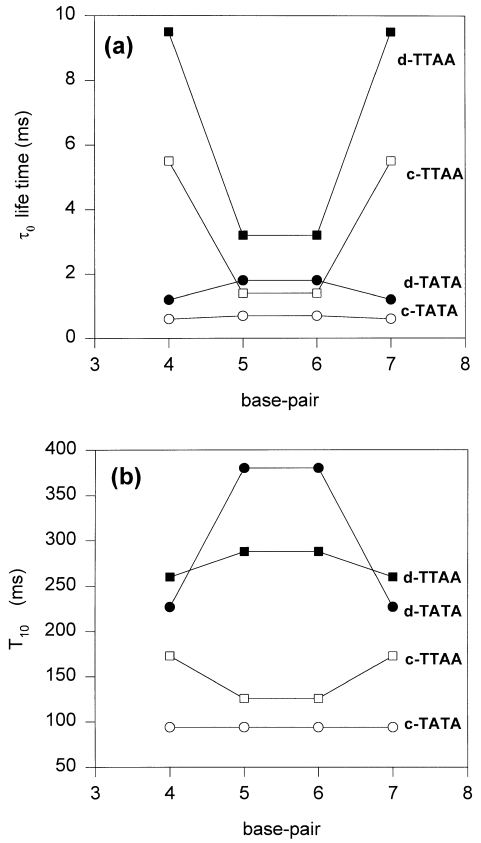

Fig. 4. The base-pair lifetimes (a) and recovery time (b) of the central four base pairs in the echinomycin–DNA complexes, TTAA-complex (c-TTAA) and TATA-complex (c-TATA), and the free DNA duplexes, TTAA-duplex (d-TTAA) and TATA-duplex (d-TATA).

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