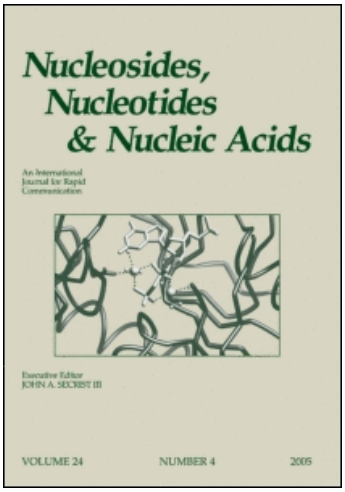

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Fast Kinetic Studies by Fluorescence Correlation Spectroscopy of Pna-Dna Interactions

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FAST KINETIC STUDIES BY FLUORESCENCE CORRELATION SPECTROSCOPY OF PNA-DNA INTERACTIONS

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ABSTRACT. The kinetics of the hybridization of fluorescently labeled Peptide nucleic acid (PNA) with its complementary single strand DNA templates and strand displacement from the same DNA duplexed with its 18-nucleotide(nt) long sequencing primer was measured. We used M13mp18 (+)ssDNA as template and the PNA molecule was 18-nt long with the identical sequence as to that of the sequencing primer of the DNA template. Kinetic studies show that hybridization was approximately 100-fold faster than the oligonucleotide-displacement at room temperature.

Introduction

Peptide nucleic acid (PNA) is an intelligently synthesized nucleic acid where the entire sugar-phosphate backbone of nucleic acid is substituted by a peptide-like N-(2-aminoethyl) glycine unit utilizing standard peptide chemistry[1,2]. PNA is a promising candidate as a gene targeting agent. It could recognize both DNA and RNA sequence specifically[3]. It has also been shown that PNA binds to duplex homopurine sequences of DNA by strand invasion[4]. Various types of PNA-nucleic acid complexes have been identified[5]. However, kinetic studies on the hybridization of PNA with single stranded nucleic acid were almost untouched. Hybridization is widely used in gene detection and

base sequencing and helps to understand the structure-function of gene expression. Kinetic studies of such processes elucidate the mechanism of formation of the structure. This type of study with PNA is the first of that kind. This could be measured in various ways, in the present paper we employed fluorescence correlation spectroscopy (FCS). With this technique the thermal fluctuations of molecules excited to fluoresce is observed and correlated. The excitation and observation are done in very small confocally defined volume elements (0.2fl.) so that fluctuations at the level of single molecule are observed[6-10]. Another advantage of FCS is that it could be applied to extremely dilute solutions (10^{-8}-10^{-15}M) and the sensititvity of detection is such that it is possible to detect single molecule[9,11]. We follow the interaction of the fluorescently labeled ligand (i.e., PNA) with the larger DNA template (single or double stranded) by measuring the correlation function that describes the diffusion of bound and free ligand in solution.

In the present paper we report the kinetic studies of hybridization and strand displacement of PNA with DNA. This helps not only in understanding various aspects of the process itself but also it shows the efficiency of the method FCS. To the best of our knowledge this is the first report of tracking the fast kinetics by studying the kinetics of hybridization and strand displacement of the 18-nt PNA primer with ssM13 DNA or duplexed with the DNA primer.

Experimental.

Tetramethylrhodamine isothiocyanate (TRITC) -labeled 18-nt long PNA-primer ("TRITC"-TGTAAAACGACGGCCAGT-NH_2) was synthesized using the standard methods[2]. The TRITC was coupled to the finished PNA oligomer on the solid support and the product was purified by reversed phase HPLC and characterized by MAGDI-TOF mass spectrometry.

M13mp18(+) ssDNA (7250 bases) and the 18-nt DNA-primer of the same sequence to that of the PNA-primer were purchased from Pharmacia (Sweden).

The experimental set-up and theoretical background of FCS have been reported earlier[6-10]. The experiments were done using the Zeiss-evotec-CONFOCOR FCS instrument. This was equipped with the built-in Axiovert 135 TV. Some experiments were also done with the indigenous arrangement that consists of an argon laser (Spectra

Physics Model 165), an epi-illuminated microscope with a water immersion objective (Zeiss Neofluar, 63 X 1.2), an avalanche photodiode (EG&G, SPCM-100 PQ) in photocounting mode as detector and a digital signal correlator (ALV Laser Vertriebsgesellschaft GmbH, ALV-5000). Data analysis was done either by using the CONFOCOR software or using non-linear least square's parameterization for calculating the normalized mean square deviation between data and model. Model used for determination of the fraction bound and its diffusion time was described elsewhere[12]. The TRITC-labeled PNA was excited at 514.5nm.

For both hybridization and oligonucleotide-displacement kinetic studies we employed the one step bimolecular kinetic model assuming the backward rate are very slow. The dissociation constant for PNA-DNA duplex is very slow hence the proposition is true. However, further work is going in our laboratory to establish the complete kinetic mechanism of oligonucleotide-displacement reaction assuming two step reactions with the formation of an intermediate.

For the kinetic measurements we used 50nM of each species. Hybridization studies were done at three different temperatures to evaluate the activation energy and to know the temperature dependence of the rate constants.

RESULTS

TRITC and PNA-labeled with TRITC had the 16000 and 13000 counts/molecule, respectively. The diffusion time of TRITC was between 0.07 and 0.08ms while that for the labeled PNA varied between 0.16 and 0.34ms (Figure 1). However, these values are very much dependent on the size of the volume element, temperature and other experimental conditions.

The formation of complex at a particular temperature with elapsed time was monitored by following the change in autocorrelation function. The translational diffusion time of the labeled PNA upon complexation with M13mp18(+) ssDNA was between 3 and 4ms (figure 1). The difference in diffusion time of the free ligand and the complex enables us to determine the fraction bound at different time without any necessity for separating them. Whether strand hybridization or oligonucleotide-displacement, in both cases we used equimolar concentrations of the interacting species. The curves of fraction

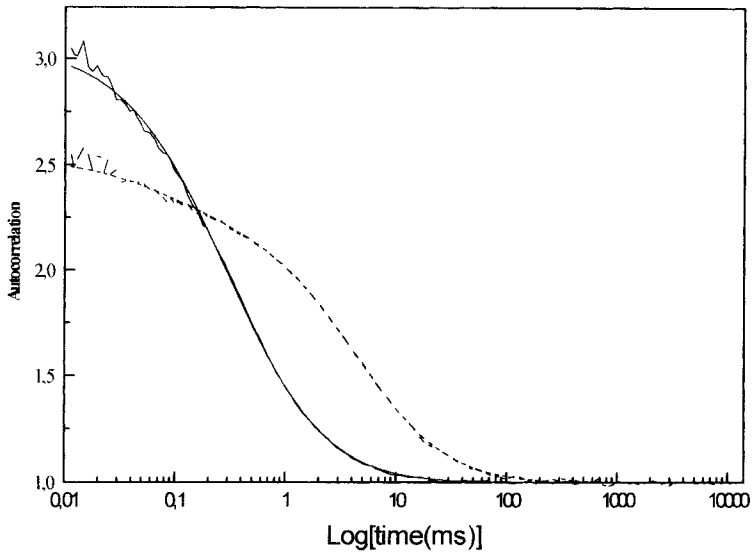

Figure 1. Autocorrelation functions of free (τ_{diff}=0.34ms, solid line) and M13-DNA-bound (τ_{diff}=4ms, dashed line) TRITC-labeled PNA

bound versus Time were fitted with the equation $f(t)=1-[1/(1+kC_0t)]$, where k is the rate constant for the association or oligonucleotide displacement and C_0 was the total concentration of the ligand and the template DNA.

Figure 2 shows the kinetics of hybridization of PNA with the DNA template at different temperatures and figure 3 shows that for the oligonucleotide-displacement. Values of kinetic parameters are listed in table 1.

DISCUSSION

The above results show that the hybridization of the PNA primer with its DNA template is very fast. The values of the rate constants are not much dependent on the temperature suggesting a low activation barrier for the association. Inset to figure 3 shows that temperature dependence of the rate constants is linear in the range discussed here and the activation energy is very low. As reported earlier that equilibrium binding constant is also very high which supports our proposition that dissociation constant for strand separation is very slow. The rate constants for hybridization are in the order 0f $10^7 M^{-1}S^{-1}$.

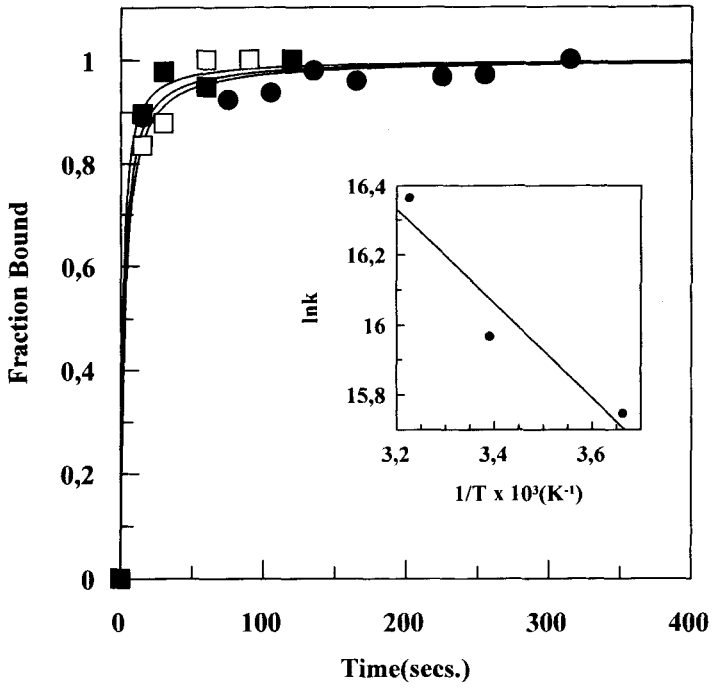

Figure 2. Change in fraction bound upon hybridization of TRITC-labeled 18nt-PNA with M13mp18(+) ssDNA at 0°C (□), 22°C (■) and 37°C (●). Solid lines are the second order fitting curves evaluated using the bimolecular 2nd order rate equation as described in the text. Energy of activation (E_{act}) = 2.8(±0.4)kCal/mol was determined from the Arrhenius plot (inset to the figure).

Table 1. Kinetic parameters for the following interactions. Uncertainty in the values is around 20%.

Interaction	Temperature (°C)	$k(M^{-1}s^{-1})$
PNA-DNA-hybridization	0	0.69×10^7
"	22	0.86×10^7
"	37	1.3×10^7
Oligonucleotide-displacement	22	4.4×10^5

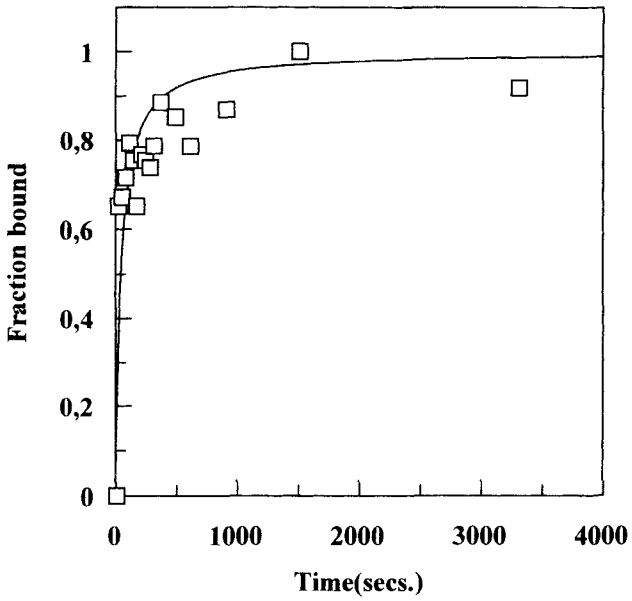

Figure 3. Time evolution of the oligonucleotide displacement interaction of TRITC-labeled 18-nt PNA with the M13-DNA duplexed with the 18nt-long DNA sequencing primer. Solid line is the second order fitting curve evaluated by the method as described in figure 2.

The results further show that the association of this PNA molecule with the short duplex DNA after displacing the third strand or formation of a triplex is roughly 100-fold slower at 22°C.

However, further studies are going in our laboratory to elucidate the detailed mechanism of oligonucleotide-displacement interaction.

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