Biochemical and Biophysical Research Communications 385 (2009) 88–93

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Single-fluorophore monitoring of DNA hybridization for investigating the effect of secondary structure on the nucleation step

Joon-Jung Jo, Min-Ji Kim, Jung-Tae Son, Jandi Kim, Jong-Shik Shin *

Department of Biotechnology, Yonsei University, Shinchon-Dong 134, Seodaemun-Gu, Seoul 120-749, South Korea

ARTICLE INFO

Article history:
Received 1 April 2009
Available online 5 May 2009

Keywords:
DNA oligonucleotide
Hybridization kinetics
Single-fluorophore measurement
Secondary structure
Nucleation
Activation energy
Salt dependence

ABSTRACT

Nucleic acid hybridization is one of the essential biological processes involved in storage and transmission of genetic information. Here we quantitatively determined the effect of secondary structure on the hybridization activation energy using structurally defined oligonucleotides. It turned out that activation energy is linearly proportional to the length of a single-stranded region flanking a nucleation site, generating a 0.18 kcal/mol energy barrier per nucleotide. Based on this result, we propose that the presence of single-stranded segments available for non-productive base pairing with a nucleation counterpart extends the searching process for nucleation sites to find a perfect match. This result may provide insights into rational selection of a target mRNA site for siRNA and antisense gene silencing.

Introduction

Nucleic acid hybridization based on Watson–Crick base-pair complementarity underlies a number of essential biological processes such as genetic information storage and transmission [1], three-dimensional folding of nucleic acids [2] and genetic regulation by non-coding RNA elements [3]. Mechanism-based understanding of the hybridization process is a prerequisite to elucidation of the relevant biological phenomena. In addition to the biological implications, recent advent of new technologies involving short oligonucleotides leads one to pay increasing attention to kinetics and thermodynamics of the nucleic acid hybridization. Typical examples are nucleic acid-based biosensors and microarrays [4,5]. In these applications, predesigned secondary structures of oligonucleotides are often coupled to dynamic functionality of the resulting devices [2]. For example, hairpin structures of molecular beacons ensure spatial proximity of end-labeled fluorophores until a complementary counterpart to a loop region separates the fluorophores by formation of a rigid duplex [6,7]. This example clearly indicates that improving performance of the nucleic acid-based devices requires a thorough understanding on how secondary structures affect hybridization kinetics.

It is generally accepted that nucleic acid hybridization is initiated by formation of a nucleation complex in which stable intermolecular base pairing between complementary regions is formed with a length of a few base pairs [1]. The nucleation complex undergoes zipping-up of unpaired regions, completing the hybridization process [8–10]. The two-step model successfully explained the hybridization kinetics for unstructured oligonucleotides (i.e., devoid of intramolecular secondary structures) [11–14]. In the case of hybridization between structured oligonucleotides, the zipping-up step involves local disruption of intramolecular base pairing. Therefore, hybridization kinetics becomes more complicated than the one for unstructured oligonucleotides. A kinetic model, to our knowledge, is not yet available to predict the effect of secondary structures on the hybridization kinetics [8–10]. To develop a reliable kinetic model, it is essential to understand how the nucleation step is influenced by intramolecular secondary structures. In this study, we have attempted to address the issue using structurally defined oligonucleotides with a varying length of a single-stranded overhang segment close to a nucleation site.

To this end, an accurate detection method should be employed to monitor the progress of hybridization reaction. Monitoring nucleic acid hybridization has been carried out using absorbance hypochromicity [15–17], fluorescence resonance energy transfer (FRET) [8,9,13,14,18–21] and surface plasmon resonance (SPR) [10,22,23]. However, there is an intrinsic drawback of these approaches that changes in the detection signal result not only from the final hybridization product but also from hybridization intermediates contributing to early detection signal. To achieve accurate kinetic analysis, it is desirable to use a detection method of which signal changes are only caused by accumulation of the final product. In this study, we monitored DNA hybridization by

* Corresponding author. Fax: +82 2 362 7265.
E-mail address: enzymo@yonsei.ac.kr (J.-S. Shin).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.04.140

measuring fluorescence changes of a single fluorescent probe end-labeled on one of the two DNA oligonucleotides. Using the single-fluorophore measurement, hybridization rate constants were determined at different temperatures and salt concentrations to investigate the effect of secondary structures on activation energy and change in the number of cations associated in a rate-determining step. From the kinetic measurements, we found that activation energy is directly dependent on the length of a single-stranded overhang that might hinder the nucleation step.

Materials and methods

Synthetic oligonucleotides and secondary structure prediction. HPLC-purified DNA oligonucleotides were purchased from IDT, Inc. (Coralville, USA). Dye labeling of strand O was performed by the supplier. DNA stock solutions (100 μM) were prepared in TE buffer (pH 8.0, 10 mM Tris base, 1 mM EDTA). Sequences for the DNA oligonucleotides were randomly generated and then manually optimized using mfold [24] to minimize undesirable intramolecular secondary structures and unfavorable intermolecular hybridization. DNA secondary structures were predicted by mfold at the conditions of 300 mM NaCl and 298 K. Secondary structure stability (ΔG) was obtained from the mfold calculation.

Kinetic measurements. All the hybridization reactions were performed in a sodium phosphate buffer (50 mM, pH 6.5). Typical reaction conditions were 5 nM strand O and 50 nM strand T (or TN) in the reaction buffer supplemented with 250 mM NaCl (100 μl working volume). Fluorescence measurements were carried out with a fluorometer equipped with a Peltier temperature controller and a photomultiplier detector (PTI Co.). Excitation and emission wavelengths were set to 496 and 517 nm, respectively (both 4 nm bandwidth). Changes in the fluorescence emission were recorded, which were used in curve fitting to a single exponential function.

Kinetic analysis. Pseudo-steady-state reaction conditions were used for kinetic analysis. The kinetic traces obtained from the fluorescence measurements were subjected to curve fitting to determine rate constants (k_{hyb}). Under the reaction conditions (i.e., $[T]_0 \gg [O]_0$ where [T] and [O] represent concentrations of strands T and O, respectively, and subscript 0 represents an initial state), the reaction follows pseudo-first-order kinetics.

$$\frac{d[O]}{dt} = -k_{hyb}[T]_0[O] \quad (1)$$

Integration of Eq. (1) yields a single exponential function whose exponent is linearly proportional to $[T]_0$ (i.e., $k_{obs} = k_{hyb}[T]_0$).

$$[O] = [O]_0 e^{-k_{obs} \cdot t} \quad (2)$$

Because both O and T–O (i.e., the final hybridization product) contribute to fluorescence emission, the total intensity with respect to time is given by

$$f(t) = \varepsilon_{T-O}[T-O] + \varepsilon_O[O], \quad (3)$$

where ε is the molar fluorescence intensity. Because [T–O] equals $[O]_0 - [O]$, Eq. (3) becomes

$$f(t) = \varepsilon_{T-O}[O]_0 + (\varepsilon_O - \varepsilon_{T-O})[O]_0 e^{-k_{obs} \cdot t} \quad (4)$$

Eq. (4) was used for curve fitting to obtain k_{obs} values, and then k_{hyb} values from linear regression between k_{obs} and $[T]_0$.

To determine activation energy, the k_{hyb} values obtained at different temperatures (15–37 °C) were fitted to the Arrhenius equation.

$$\ln k_{hyb} = \ln A - E_a/RT \quad (5)$$

Table 1
DNA sequences for the oligonucleotides to examine effect of hybridization state on the fluorescence intensity.

O	5′-CGATTCAAGCGGTTTGGCGTG-Oregon Green 488-3′
C[a]	5′-CACGCCAAACCGCTTGAATCG-3′
T1[b]	5′-GACTCGCACATCAGTTACCCGCGGAGAGGCTCACGCCAAACCGCTTGAATCG-3′
I	5′-AGCCTCTCCG-3′

[a] Strand C is a perfect complement to strand O.
[b] The single-underlined and double-underlined segments in strand T1 are complementary to strand I and strand O, respectively.

Results

Monitoring DNA hybridization using a single fluorophore

It is known that fluorescence intensity is dependent on hydrophobic environments of a fluorophore [25]. To examine how the hybridization state affects emission intensity of a fluorophore labeled on a DNA oligonucleotide, we measured changes in the fluorescence emission of Oregon Green 488 end-labeled at the 3′-end of strand O upon hybridization with complementary strands shown in Table 1. The fluorescence emission from strand O decreased by 58% at the maximum emission wavelength (λ_{max} = 517 nm) upon formation of a 21-bp duplex with strand C (Fig. 1). Hybridization between strands O and T1 resulted in a 21-bp duplex with a 31-base single-stranded overhang, leading to an increase in fluorescence emission by 31% compared with the O–C duplex. Intriguingly, further hybridization of the O–T1 complex with strand I led to even higher fluorescence emission than that of strand O. In the O–T1–I trimolecular complex, both regions flanking the fluorophore are double-stranded. These results clearly show that emission intensity of a fluorophore labeled on a DNA oligonucleotide is highly sensitive to changes in the local hybridization state. The fluorescence changes shown in Fig. 1 were large enough to allow real-time monitoring of the hybridization reaction, leading us to undertake kinetic measurements using the single fluorophore.

Time-course monitoring of the hybridization reaction between strands O and T1 was carried out by measuring fluorescence intensity of Oregon Green 488 labeled on strand O (Fig. 2). Because of the lower fluorescence emission of the O–T1 complex than that

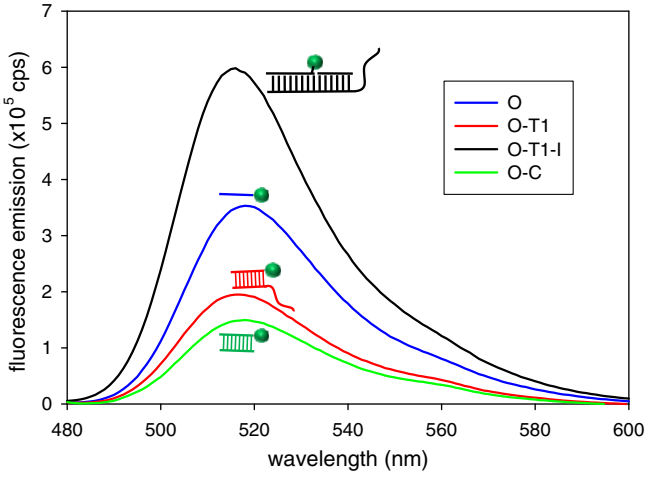

Fig. 1. Changes in fluorescence emission of strand O depending on the hybridization state. Concentrations of the oligonucleotides were 10 nM. Excitation wavelength was set to 470 nm. The green sphere represents Oregon Green 488 labeled at the 3′-end of strand O.