Biochimie 94 (2012) 1032–1040

Contents lists available at SciVerse ScienceDirect

Biochimie

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

Research paper

Chemical modification of triplex-forming oligonucleotide to promote pyrimidine motif triplex formation at physiological pH

Hidetaka Torigoe [a,*], Osamu Nakagawa [b], Takeshi Imanishi [b], Satoshi Obika [b], Kiyomi Sasaki [a]

[a] Department of Applied Chemistry, Faculty of Science, Tokyo University of Science, 1-3 Kagurazaka, Shinjuku-ku, Tokyo 162-8601, Japan
[b] Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 21 June 2011
Accepted 4 January 2012
Available online 9 January 2012

Keywords:
Triplex formation
Chemical modification of triplex-forming oligonucleotide
Thermodynamic parameters
Rigidity
Hydration
Nuclease resistance

ABSTRACT

Extreme instability of pyrimidine motif triplex DNA at physiological pH severely limits its use in wide variety of potential applications, such as artificial regulation of gene expression, mapping of genomic DNA, and gene-targeted mutagenesis *in vivo*. Stabilization of pyrimidine motif triplex at physiological pH is, therefore, crucial for improving its potential in various triplex-formation-based strategies *in vivo*. To this end, we investigated the effect of 3′-amino-2′-*O*,4′-*C*-methylene bridged nucleic acid modification of triplex-forming oligonucleotide (TFO), in which 2′-O and 4′-C of the sugar moiety were bridged with the methylene chain and 3′-O was replaced by 3′-NH, on pyrimidine motif triplex formation at physiological pH. The modification not only significantly increased the thermal stability of the triplex but also increased the binding constant of triplex formation about 15-fold. The increased magnitude of the binding constant was not significantly changed when the number and position of the modification in TFO changed. The consideration of the observed thermodynamic parameters suggested that the increased rigidity of the modified TFO in the free state resulting from the bridging of different positions of the sugar moiety with an alkyl chain and the increased hydration of the modified TFO in the free state caused by the introduction of polar nitrogen atoms may significantly increase the binding constant at physiological pH. The study on the TFO viability in human serum showed that the modification significantly increased the resistance of TFO against nuclease degradation. This study presents an effective approach for designing novel chemically modified TFOs with higher binding affinity of triplex formation at physiological pH and higher nuclease resistance under physiological condition, which may eventually lead to progress in various triplex-formation-based strategies *in vivo*.

1. Introduction

In recent years, triplex nucleic acid has attracted considerable interest because of its possible biological functions *in vivo* and its wide variety of potential applications, such as artificial regulation of gene expression by antigene technology, mapping of genomic DNA, and gene-targeted mutagenesis [1–5]. A triplex nucleic acid is usually formed through the sequence-specific interaction of a single-stranded homopyrimidine or homopurine triplex-forming oligonucleotide (TFO) with the major groove of a homopurine–homopyrimidine stretch in duplex DNA [3,4]. In the pyrimidine motif triplex, a homopyrimidine TFO binds parallel to the homopurine strand of the target duplex by Hoogsteen hydrogen bonding to form T•A:T and C^+•G:C base triplets [3,4]. On the other hand, in the purine motif triplex, a homopurine TFO binds antiparallel to the homopurine strand of the target duplex by reverse Hoogsteen hydrogen bonding to form A•A:T (or T•A:T) and G•G:C base triplets [3,4].

Because protonation of the cytosine bases in a homopyrimidine TFO is required to enable binding with the guanine bases of the G:C target duplex, the formation of the pyrimidine motif triplex requires an environment with acidic pH, and is thus extremely unstable at physiological neutral pH [6–8]. On the other hand, the pH-independent formation of the purine motif triplex is possible at physiological neutral pH. However, purine motif triplex formation is severely inhibited by physiological concentrations of certain monovalent cations, especially K^+ ions [9,10]. The undefined association between K^+ and the guanine-rich homopurine TFO has been considered to explain this inhibitory effect [9,10]. Thus, the stabilization of the pyrimidine motif triplex at physiological neutral pH is necessary for improving its potential in various

Abbreviations: 3′-amino-2′,4′-BNA, 3′-amino-2′-*O*,4′-*C*-methylene bridged nucleic acid; CD, circular dichroism; EMSA, electrophoretic mobility shift assay; HPLC, high-performance liquid chromatography; ITC, isothermal titration calorimetry; TFO, triplex-forming oligonucleotide.

* Corresponding author. Tel.: +81 3 5228 8259; fax: +81 3 5261 4631.
E-mail address: htorigoe@rs.kagu.tus.ac.jp (H. Torigoe).

0300-9084/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2012.01.003

triplex-formation-based strategies. Replacement of the cytosine bases in a homopyrimidine TFO with 5-methylcytosine [7,11–13] or other chemically modified base analogues [14–18], and conjugation of different DNA intercalators to TFO [19,20] have been used to overcome the requirement of an acidic pH for the pyrimidine motif triplex formation and to stabilize the pyrimidine motif triplex at physiological neutral pH.

We first synthesized and developed a new class of chemical modifications of nucleic acids, 3′-amino-2′-*O*,4′-*C*-methylene bridged nucleic acid (3′-amino-2′,4′-BNA) (Fig. 1a), in which 2′-O and 4′-C of the sugar moiety were bridged with the methylene chain, and 3′-O was replaced by 3′-NH [21,22]. The thermal stability of the triplex with 3′-amino-2′,4′-BNA modified TFO at physiological neutral pH was much higher than that with the corresponding natural phosphodiester TFO, which was shown by the UV melting of the dissociation of the triplex [21,22]. However, the formation of the triplex involving 3′-amino-2′,4′-BNA modified TFO at physiological neutral pH has not yet been well-characterized. To explore the possibility of the application of 3′-amino-2′,4′-BNA modified TFO to various triplex-formation-based strategies *in vivo*, the investigation of the formation of the triplex involving 3′-amino-2′,4′-BNA modified TFO at physiological neutral pH may be more important than that of the dissociation of the same triplex. In addition, the mechanistic explanation for the 3′-amino-2′,4′-BNA modification-mediated triplex stabilization at physiological neutral pH remains to be provided. Therefore, we have examined the effect of the 3′-amino-2′,4′-BNA modification of TFO on pyrimidine motif triplex formation with another base sequence at physiological neutral pH. The thermodynamic effect of the 3′-amino-2′,4′-BNA modification on the pyrimidine motif triplex formation between a 23-base pair homopurine-homopyrimidine target duplex (Pur23A•Pyr23T) (Fig. 1b) and its specific 15-mer unmodified homopyrimidine TFO (Pyr15T) (Fig. 1b) or each of 3′-amino-2′,4′-BNA modified homopyrimidine TFO (Pyr15BNANP7-1, Pyr15BNANP7-2, Pyr15BNANP5-1, and Pyr15BNANP5-2) (Fig. 1b) has been analyzed by the electrophoretic mobility shift assay (EMSA) [23–29], UV melting, and isothermal titration calorimetry (ITC) [24–26,28,30–33]. To examine the effect of the modified positions, Pyr15BNANP7-1 and Pyr15BNANP7-2 contain one modification every 2 nucleotides starting from the first and second positions at the 5′-terminal, respectively. Pyr15BNANP5-1 and Pyr15BNANP5-2 contain one modification every 3 nucleotides starting from the first and second positions at the 5′-terminal, respectively. To explore the possibility of the application of 3′-amino-2′,4′-BNA modified TFOs *in vivo*, the resistance of the unmodified and 3′-amino-2′,4′-BNA modified TFOs against nuclease degradation in human serum has been also investigated by native polyacrylamide gel electrophoresis and anion-exchange high-performance liquid chromatography (HPLC). We found that the 3′-amino-2′,4′-BNA modification of TFO increased the binding constant for the pyrimidine motif triplex formation at physiological neutral pH about 15-fold. We also observed that the nuclease resistance of the 3′-amino-2′,4′-BNA modified TFOs was significantly higher than that of the unmodified TFO. The mechanism of the 3′-amino-2′,4′-BNA modification to promote the pyrimidine motif triplex formation will be discussed. This information will present an effective approach for designing novel chemically modified TFOs with higher binding affinity of the pyrimidine motif triplex formation at physiological neutral pH.

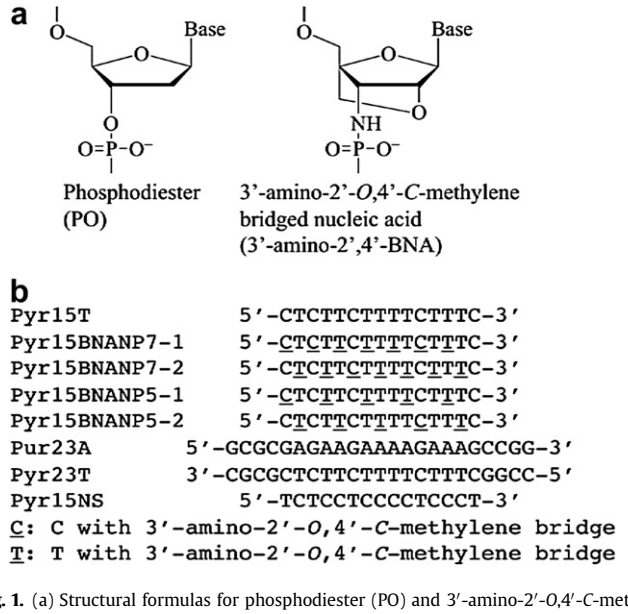

Fig. 1. (a) Structural formulas for phosphodiester (PO) and 3′-amino-2′-*O*,4′-*C*-methylene bridged nucleic acid (3′-amino-2′,4′-BNA)-modified backbones. (b) Oligonucleotide sequences for the target duplex (Pur23A•Pyr23T), the specific TFOs (Pyr15T, Pyr15BNANP7-1, Pyr15BNANP7-2, Pyr15BNANP5-1, and Pyr15BNANP5-2), and the nonspecific oligonucleotide (Pyr15NS).

2. Materials and methods

2.1. Preparation of oligonucleotides

We synthesized 23-mer complementary oligonucleotides for the target duplex, Pur23A and Pyr23T (Fig. 1b), a 15-mer unmodified homopyrimidine TFO specific to the target duplex, Pyr15T (Fig. 1b), and a 15-mer nonspecific homopyrimidine oligonucleotide, Pyr15NS (Fig. 1b), on an ABI DNA synthesizer using the solid-phase cyanoethyl phosphoramidite method; we then purified them by reverse-phase HPLC on a Wakosil DNA column. The 15-mer 3′-amino-2′,4′-BNA modified homopyrimidine TFOs specific to the target duplex, Pyr15BNANP7-1, Pyr15BNANP7-2, Pyr15BNANP5-1, and Pyr15BNANP5-2 (Fig. 1b), were synthesized and purified as described previously [21,22]. The concentration of all oligonucleotides was determined by UV absorbance. Complementary strands, Pur23A and Pyr23T, were annealed by heating up to 90 °C and then gradually cooling to room temperature. The annealed sample was applied to a hydroxyapatite column (BIORAD Inc.) to remove unpaired single strands. The concentration of the duplex DNA (Pur23A•Pyr23T) was determined by UV absorption, considering that an absorbance of 1 at 260 nm corresponds to a concentration of 50 μg/mL of DNA, with a M_r of 15180.

2.2. EMSA

EMSA experiments were performed as previously described, by 15% native polyacrylamide gel electrophoresis [24–29]. In a 9 μL aliquot of the reaction mixture, ^{32}P-labeled Pur23A•Pyr23T duplex (~1 nM) was mixed with increasing concentrations of the specific TFO (Pyr15T, Pyr15BNANP7-1, Pyr15BNANP7-2, Pyr15BNANP5-1, or Pyr15BNANP5-2) and the nonspecific oligonucleotide (Pyr15NS) in buffer [50 mM Tris-acetate (pH 7.0), 100 mM NaCl, and 10 mM $MgCl_2$]. Pyr15NS was added to achieve equimolar concentrations of TFO in each lane as well as to minimize the adhesion of the DNA (duplex and TFO) to plastic surfaces during incubation and subsequent losses during processing. After 6 h incubation at 37 °C, 2 μL of 50% glycerol solution containing bromophenol blue was added without changing the pH and salt concentrations of the reaction mixtures. Samples were then directly loaded onto a 15% native polyacrylamide gel prepared in buffer [50 mM Tris-acetate (pH 7.0) and 10 mM $MgCl_2$], and electrophoresis was performed at 8 V/cm for 16 h at 4 °C.