Stopped-flow kinetics of locked nucleic acid (LNA)–oligonucleotide duplex formation: studies of LNA–DNA and DNA–DNA interactions

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The locked nucleic acid (LNA) monomer is a conformationally restricted nucleotide analogue with an extra 2'-O,4'-C-methylene bridge added to the ribose ring. Oligonucleotides that contain LNA monomers have shown greatly enhanced thermal stability when hybridized to complementary DNA and RNA and are considered most promising candidates for efficient recognition of a given mixed sequence in a nucleic acid duplex and as an antisense molecule. Here the kinetics and thermodynamics of a series of oligonucleotide duplex formations of DNA–DNA and DNA–LNA octamers were studied using stopped-flow absorption measurements at 25 °C and melting curves. The reactions of the DNA octamer 5'-CAGGAGCA-3' with its complementary DNA octamer 5'-TGCTCCTG-3', and with the LNA octamers 5'-T^LGCTCCTG-3' (LNA-1), 5'-T^LGCT^LCCTG-3'

(LNA-2) and 5'-T^LGCT^LCCT^LG-3' (LNA-3), containing respectively one, two or three thymidine 2'-O,4'-C-methylene-(D-ribofuranosyl) nucleotide monomers, designated T^L, were studied. In all cases were seen fast second-order association reactions with $k_{\text{obs}} = 2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. At 25 °C the dissociation constants of the duplexes obtained from melting curves were: DNA–DNA, 10 nM; DNA–LNA-1, 20 nM; DNA–LNA-2, 2 nM; and DNA–LNA-3, 0.3 nM; thus the greatly enhanced duplex stability induced by LNA is confirmed. Since the association rates were all equal this increase in stability is due to slower rates of dissociation of the complexes.

Key words: DNA oligomer, off-rates, on-rates, thermodynamics.

INTRODUCTION

The locked nucleic acid (LNA) monomer is a conformationally restricted nucleotide analogue with an extra 2'-O,4'-C-methylene bridge added to the ribose ring (Figure 1). LNA has been defined as an oligonucleotide containing one or more 2'-O,4'-C-methylene-(D-ribofuranosyl) nucleotide monomers [1–3]. Such oligonucleotides that contain LNA monomers have shown stability towards 3'-exonucleolytic degradation and greatly enhanced thermal stability when hybridized to complementary DNA and RNA [1–5]. In fact, in comparison with other high-affinity nucleic acid mimics that have been synthesized, e.g. peptide nucleic acids (PNAs) [6,7], hexitol nucleic acids (HNAs) [8,9] and 2'-fluoro N³-phosphoramidates [10], LNA displays unprecedented binding affinities [1–5]. Since fluorescein-labelled LNA can also be transferred into MCF-7 breast cancer cells [11], the LNA molecule is a most promising candidate for efficient recognition of a given mixed sequence in a nucleic acid duplex and as an antisense molecule.

In order to obtain a more detailed understanding of LNA-hybridization reactions we have studied the stopped-flow kinetics and thermodynamics of a series of reactions of DNA and LNA octamers.

EXPERIMENTAL

Oligonucleotides

The DNA octamer 5'-TGCTCCTG-3' (DNA-T; $\epsilon_{260} = 65700 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and the complementary DNA octamer 5'-CAGGAGCA-3' (DNA-A; $\epsilon_{260} = 84100 \text{ M}^{-1} \cdot \text{cm}^{-1}$) were synthesized and purified using standard phosphoramidite chemistry

as described in [12]. Also, molar absorptivities were calculated as described in [12].

The LNA-containing octamers 5'-T^LGCTCCTG-3' (LNA-1), 5'-T^LGCT^LCCTG-3' (LNA-2) and 5'-T^LGCT^LCCT^LG-3' (LNA-3), containing one, two or three LNA-T^L monomers respectively, where T^L is the thymidine 2'-O,4'-C-methylene-(D-ribofuranosyl) nucleotide monomer, were synthesized and purified as described in [2]. Their molar absorptivity, $\epsilon_{260} = 65700 \text{ M}^{-1} \cdot \text{cm}^{-1}$, was calculated assuming identical absorptivities for LNA and DNA monomeric nucleotides. The oligonucleotides were lyophilized and stored at –20 °C.

Stopped-flow kinetic experiments

Stock solutions (approx. 100 μM) of the oligonucleotides were made in water. Their concentrations were accurately determined from absorbance measurements at $\lambda = 260 \text{ nm}$ in a Perkin–Elmer λ -17 spectrophotometer. Dilutions were made in a buffer, pH 7.0, containing 0.01 M Pipes, 0.01 M MgCl₂ and 0.1 M NaCl. The reactions of DNA–T, LNA-1, LNA-2 and LNA-3 with DNA–A were studied using a series of equal concentrations of the reactants in the range 10–0.5 μM . The stopped-flow kinetic experiments were performed at 25 °C using a Hi-Tech Scientific PQ/SF-53 spectrophotometer equipped with a high-intensity xenon arc lamp. After rapid mixing (approx. 10^{-3} s) of equal volumes of the reactants, the time course of the absorbance ($\lambda = 260 \text{ nm}$) was recorded; 400 pairs of data were taken in each experiment. Data sets from three or four experiments performed under identical conditions were averaged. The averaged stopped-flow data were fitted to several non-linear analytical equations using the Hi-Tech software. In all cases the expression describing a second-order reaction with equal concentrations of the reactants resulted in the best fit.

Abbreviations used: LNA, locked nucleic acid; T^L, thymidine 2'-O,4'-C-methylene-(D-ribofuranosyl) nucleotide monomer; T_m , melting temperature.

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Figure 1 The chemical structure of a DNA monomer and two representations of the chemical structure of a LNA

Melting experiments

The melting of the duplexes DNA-A–DNA-T, DNA-A–LNA-1, DNA-A–LNA-2 and DNA-A–LNA-3 were studied by measuring absorbance ($\lambda = 260$ nm) as a function of temperature from 10 to 65 °C, with an increase of 1 °C/min in a Perkin-Elmer λ -40 spectrophotometer equipped with a Peltier element controlling the temperature. Mixtures containing 1.5 μ M of each of the reactants in the Pipes buffer, pH 7.0, were used.

RESULTS

Theory

Duplex formation of oligonucleotides is generally accepted [13] as a process involving two main events, initial formation of a nucleation complex followed by the sealing of the duplex. Thus the expected reaction scheme for the formation of a duplex, D, of two complementary DNA oligomers, DNA-A and DNA-T, may be depicted as:

where IC is a nucleation complex and the temperature-dependent overall dissociation constant, K_D , is given by eqn. (2):

$$K_D = (k_{-1} \cdot k_{-2}) / (k_1 \cdot k_2) = [\text{DNA-A}_{\text{eq}}][\text{DNA-T}_{\text{eq}}] / [\text{D}_{\text{eq}}] \quad (2)$$

where eq designates the concentrations at equilibrium.

It is also generally accepted [13] that the intermediate, initial association complex is unstable, with a marked tendency to either dissociate or seal, depending on the temperature. Thus it is always expected to be present in low amounts compared with the monomers or the duplex. In other words it is expected that the dissociation constant of IC, K_1 (units, molar con-

Figure 2 A typical result from a stopped-flow experiment

The time course shown is an average of three traces, each obtained after mixing of LNA-3 and DNA-A, at 25 °C. Final concentrations, 2.18 μ M each.

centration), $K_1 = k_{-1}/k_1$, is always large compared with concentrations normally used (μ M–mM range), and thus $K_2 = k_{-2}/k_2 \ll 1$ when the equilibrium is in favour of duplex formation, i.e. at temperatures below the melting temperature (T_m). In any case, in experiments measuring the change of absorbance occurring when a duplex forms or dissociates it seems reasonable to assume that the IC complex is silent, i.e. is not contributing significantly to the signal change and that the changes stem first of all from the formation or disappearance of D. Thus

$$dA_{260}/dt \propto dD/dt = k_2[\text{IC}] - k_{-2}[\text{D}] \quad (3)$$

If equal initial concentrations of DNA-A and DNA-T are used, C_0 is the initial concentration and $[C]$ their concentration at time t , mass conservation requires

$$C_0 - [\text{D}] = [\text{IC}] + [C] \cong [C]$$

and

$$\begin{aligned} dC/dt &= k_1[C]^2 - k_{-1}[\text{IC}] \\ &= k_1[C]^2(1 + K_1[\text{IC}]/[C]^2) \\ &\cong k_1[C]^2 \cong -dD/dt \end{aligned} \quad (4)$$

Second-order kinetics are usually assumed to indicate that the first step is slow and the second fast, and that the observed rate constant is $k_{\text{obs}} = k_1$. This may well be the case. But if the first step is fast and the second step slow, so that equilibrium prevails in the first step in the time range of the slow process, we would also get second-order kinetics as long as $C_0 \ll K_1$. In this case, $[C]^2 = [\text{IC}]K_1$ and $[C]^2/K_1 + [C] = C_0 - [\text{D}]$, which lead to

$$\begin{aligned} dD/dt &= -d[C]/dt(1 + 2[C]/K_1) \\ &= [(k_2 + k_{-2})/K_1][C]^2 - k_{-2}(C_0 + [C]) \end{aligned}$$

or

$$-d[C]/dt = \{[k_2(1 + K_2)/K_1][C]^2 - k_{-2}(C_0 + [C])\} / (1 + 2[C]/K_1) \quad (5)$$

With $k_{-2} \ll k_2$ ($K_2 \ll 1$) and the denominator $\cong 1$ for C_0 (and thus also $[C] \ll K_1$, eqn. (5) leads to second-order kinetics with $k_{\text{obs}} \cong k_2/K_1$.

Stopped-flow kinetic results

A series of stopped-flow experiments recording the time courses of the change of absorbance at $\lambda = 260$ nm after fast ($\cong 10^{-3}$ s) mixing of equal concentrations of DNA-A and DNA-T or one of the LNAs were performed. A typical result is shown in Figure 2. The actual trace is an average of three, each obtained after mixing of LNA-3 and DNA-A (final concentrations, 2.18 μ M

Table 1 Observed second-order rate constants, k_{obs} ($\text{M}^{-1} \cdot \text{s}^{-1}$), of reactions of DNA-A with DNA-T, LNA-1, LNA-2 or LNA-3 at 25 °C

The k_{obs} values are from a series of stopped-flow experiments using equal concentrations, $[C_0]$, of the reactants.

DNA-T		LNA-1		LNA-2		LNA-3	
$[C_0]$ (μM)	k_{obs} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$[C_0]$ (μM)	k_{obs} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$[C_0]$ (μM)	k_{obs} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$[C_0]$ (μM)	k_{obs} ($\text{M}^{-1} \cdot \text{s}^{-1}$)
4.4	$(2.1 \pm 0.08) \times 10^7$	2.35	$(2.0 \pm 0.09) \times 10^7$	2.3	$(1.8 \pm 0.16) \times 10^7$	2.18	$(2.6 \pm 0.24) \times 10^7$
2.2	$(2.0 \pm 0.14) \times 10^7$	2.35	$(2.1 \pm 0.20) \times 10^7$	2.3	$(2.0 \pm 1.4) \times 10^7$	2.18	$(1.5 \pm 0.65) \times 10^7$
1.1	$(2.1 \pm 0.09) \times 10^7$	1.18	$(1.8 \pm 0.11) \times 10^7$	1.15	$(2.9 \pm 0.53) \times 10^7$	1.09	$(2.4 \pm 0.2) \times 10^7$
0.55	$(2.2 \pm 0.36) \times 10^7$	0.59	$(2.6 \pm 0.51) \times 10^7$	0.58	$(2.1 \pm 0.32) \times 10^7$	0.55	$(2.4 \pm 0.36) \times 10^7$
0.28	$(2.5 \pm 0.88) \times 10^7$			0.29	$(5 \pm 3) \times 10^7$	0.27	$(3.5 \pm 0.62) \times 10^7$

Table 2 Observed rate constants and equilibrium data

Shown are the mean values of the data presented in Table 1 with S.D. ± 10 –20%. nd, not determined.

Reactants	Temperature (°C)	k_{obs} ($\text{M}^{-1} \cdot \text{s}^{-1}$)	K_D (nM)	T_m (°C; 1.5 μM)
DNA-A + DNA-T	25	2.0×10^7	10	35
DNA-A + LNA-1	25	1.9×10^7	20	34
DNA-A + LNA-2	25	2.1×10^7	2	40
DNA-A + LNA-3	25	2.4×10^7	0.3	45
DNA-A + DNA-T	10	1.5×10^7	nd	35

Figure 3 A representative example of the melting curves

The curve illustrated was obtained with a mixture of 1.5 μM each of LNA-3 and DNA-A. For further thermodynamic analysis, K_D values were calculated from the fractions of duplex, $1 - \alpha$, and of monomers, α , present at various temperatures: K_D (in μM) = $1.5 \times [\alpha^2/(1 - \alpha)]$. The ordinate represents the relative change of absorbance observed. t , temperature.

each). The rate constants, k_{obs} , obtained from fits of a second-order rate equation to the data are listed in Table 1. In general, no other rate equation showed good fits. Within the bounds of experimental error a constant value of k_{obs} was obtained in the concentration range used, which covers approximately an order of magnitude (5–0.3 μM). The first half-time thus changes approximately 10 times, and is not a constant as it would be if the reactions were first order. Table 2 summarizes the results.

Melting curves

Figure 3 illustrates a representative example of the melting curves obtained from samples containing 1.5 μM each of DNA-A and

Figure 4 Thermodynamic analysis according to the Gibbs–Helmholtz equation of K_D values

$\log_{10} K_D$ is plotted as a function of the reciprocal temperature ($1/T$, K^{-1}). DNA-A reacted with DNA-T ($\square$), LNA-1 ($\circ$), LNA-2 ($\blacksquare$) and LNA-3 ($\triangle$).

Table 3 Standard molar thermodynamic data for duplex dissociation, 25 °C

ΔH° values are from Figure 4, S.D. ± 5 –8%.

Dissociating duplex	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (kJ/Kmol)
DNA-T–DNA-A	45	251	0.69
LNA-1–DNA-A	44	254	0.7
LNA-2–DNA-A	50	268	0.73
LNA-3–DNA-A	55	302	0.83

DNA-T or one of the LNAs. The T_m values obtained are listed in Table 2. The lower line on Figure 3 represents 100% duplex and the upper line represents 0% duplex, or 100% monomers. The overall K_D values were obtained at various temperatures from the proportions of monomers and duplex present. Figure 4 shows the analysis according to the Gibbs–Helmholtz equation of the K_D values. Table 3 summarizes the thermodynamic results. The K_D values obtained at 25 °C are included in Table 2.

DISCUSSION

Oligonucleotides that contain LNA monomers have shown greatly enhanced thermal stability when hybridized to complementary DNA and RNA [1–5]. This is also confirmed here,

since the oligonucleotides 5'-T¹GCT¹CCTG-3' (LNA-2) and 5'-T¹GCT¹CCT¹G-3' (LNA-3), which contain two and three locked nucleotide monomers respectively, showed a significantly higher T_m value for their duplexes with the complementary DNA, 5'-CAGGAGCA-3' (DNA-A), compared with that of the corresponding DNA-T (Table 2). This is also reflected in the overall K_D values obtained at 25 °C, which correspond with standard Gibbs energy (ΔG°) values of -50 and -55 kJ/mol for the formation of LNA-2 and LNA-3 duplexes with DNA-A, whereas the DNA-T-DNA-A and LNA-1-DNA-A duplexes show standard ΔG° values of formation, -45 and -44 kJ/mol respectively (at 25 °C; Table 3). Almost no effects are seen when only one LNA monomer was present in the 5'-terminal position, 5'-T¹GCTCCTG-3' (LNA-1). Apparently a terminal LNA monomer is not able to induce similar stabilizing conformational changes in the neighbouring unmodified deoxynucleotide monomers to an internally positioned LNA monomer [14]. The ΔH° and ΔS° values obtained for DNA-A-DNA-T seem comparable with literature values (e.g. [15]) for the dissociation of CCGG and ACCGGU dimers; $\Delta H^\circ = 143$ kJ/mol for the tetramer and 249 kJ/mol for the hexamer, and $\Delta S^\circ = 400$ J/Kmol for the tetramer and 688 J/Kmol for the hexamer [15]. The introduction of internal LNAs shows marked increases of these values, particularly for ΔH° , compared with those of the pure DNA duplex (Table 3); this is in accordance with the ability of LNAs to form the most stable Watson-Crick base-pairing system yet developed [16].

The stopped-flow kinetics of the formation of complementary duplexes showed second-order reactions and large and equal second-order rate constants ($k_{\text{obs}} = 2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) for the reactions of DNA-A with DNA-T, as well as with LNA-1, LNA-2 and LNA-3 (Table 2). The rate constant for a diffusion-controlled process, in which a reaction occurs at every encounter, has an upper limit of $4 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ in water at 25 °C, but an estimate of the diffusion limit further requires a fractional factor to account for the fact that many encounters occur at non-productive sites, e.g. backbone-to-backbone, base-to-backbone and wrong base-to-base encounters. Since the actual ratio of the limit value and the k_{obs} value is just 200, we find it reasonable to assume that we are dealing with a diffusion-controlled process, in which each correct base-to-base encounter leads to a reaction. This also helps explain that no differences are seen in the rates of the reactions of the LNAs and the DNA-T with DNA-A, of which the same fractional factor must be expected. Thus the results are in agreement with a rate-determining, but diffusion-controlled, association reaction governed by $k_{\text{obs}} = k_1$ followed by fast sealing of the duplex. The actual rate of the association is rather slow because it is concentration-dependent and low concentrations in the μM range must be used. From the values of the overall K_D at 25 °C (Table 2) the overall dissociation rate constant $k_{\text{off}} = k_{-1} \cdot k_{-2} / k_2$ may be calculated. The values obtained are: DNA-T-DNA-A, $k_{\text{off}} = 0.2 \text{ s}^{-1}$; LNA-1-DNA-A, $k_{\text{off}} = 0.4 \text{ s}^{-1}$; LNA-2-DNA-A, $k_{\text{off}} = 0.04 \text{ s}^{-1}$ and LNA-3-DNA-A, $k_{\text{off}} = 0.006 \text{ s}^{-1}$. The high stability of the LNA-containing duplexes as compared with the DNA duplex stems from slower dissociation, not from faster formation. As indicated above the theory does not exclude second-order kinetics

with $k_{\text{obs}} = k_2 / K_1 (\text{M}^{-1} \cdot \text{s}^{-1})$, but a rate-determining k_2 , i.e. $k_2 < (k_1[C_0] + k_{-1})$, requires $k_1 > k_{\text{obs}} = 2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and a fractional factor much less than 200. This seems unlikely. We therefore conclude that the formation of DNA-DNA and DNA-LNA duplexes observed here at 25 °C involves a rate-determining association reaction followed by fast sealing of the duplexes.

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