Journal of Thermal Analysis and Calorimetry, Vol. 56 (1999) 1177-1184

THERMODYNAMICS OF A 24-MER TRIPLE HELIX FORMATION AND STABILITY

C. Giancola[1], *A. Buono*[1], *G. Barone*[1*], *L. De Napoli*[2], *D. Montesarchio*[2], *D. Palomba*[2] *and G. Picciallí*[2]

[1]Department of Chemistry, University 'Federico II' of Naples, Via Mezzocannone 4 80134 Naples
[2]Department of Organic Chemistry and Biochemistry, University 'Federico II' of Naples Via Mezzocannone 16, 80134, Naples, Italy

Abstract

In this work we report a thermodynamic characterization of stability and melting behaviour of two 24-mer DNA triplexes. The third strand, that binds the Watson-Crick double helix with Hoogsteen hydrogen bonds, contains 3'-3' phosphodiester junction that determines the polarity inversion. The target double helix is composed of adjacent and alternate fragments of oligopurine-oligopyrimidine tracts. The two helices differ from the substitution of the cytosine, involved in the junction, with the thymine. Calorimetric data reported here provide a quantitative measure of the influence of pH and base modification on the stability of a DNA triplex.

Keywords: differential scanning calorimetry, DNA triple helix, oligonucleotides, stability

Introduction

The existence of DNA triple helix structure is known since 1957 [1], four years after the discovery of the DNA double helix structure [2] but only recently the triple-helical forms of DNA have received the due attention. This renewed interest arises from: i) the discovery of intramolecular triplexes, called H–DNAs, which are formed in homopurine-homopyrimidine regions of supercoiled plasmid [3]; ii) the potential biomedical applications of triple helices in order to control gene expression at both transcriptional and replication levels [4].

Canonical triple helix formation relies upon hydrogen bonding interaction between a homopyrimidine oligonucleotide and homopurine-homopyrimidine sequence already engaged in Watson-Crick hydrogen bonds. The third strand must be located in the major groove of the double helix that is forming Hoogsteen hydrogen bonds [5] with the purine strand of the duplex. The formation of the triad C•G×C requires the protonation of the N3 of cytosine in the third strand.

The oligonucleotides that interact with target sequence on duplex DNA are often called TFOs, acronym of Triplex Forming Oligonucleotides. TFOs might be univer-

* Author for correspondence: e-mail: barone@chemna.dichi.unina.it

1418–2874/99/ $ 5.00

Akadémiai Kiadó, Budapest
Kluwer Academic Publishers, Dordrecht

sal drugs that exhibit specific sequence-recognition of duplex DNA. The 'antigene strategy' is based on highly sequence specific recognition of DNA by TFOs. Under physiological conditions, TFOs must be selective and their binding strong enough to inhibit the normal functioning of the target gene. Furthermore, TFOs must be relatively long (15–17 bases) to significantly affect genetic processes but so long homopurine-homopyrimidine stretches are infrequent.

In order to increase the number of potential targets for triplex formation in natural DNAs, many research groups [6–8] have designed alternate strand triplex; the third strand consists of adjacent homopurine and homopyrimidine blocks binding purines on alternate strands of the duplex. The strand switch can occur preserving or inverting the chain polarity. In the last case a convenient linker must be introduced for the appropriate inversion of polarity, i.e. a 3'-3' or 5'-5' internucleoside junction, which assures for both 5'- and 3'-ends the required orientation.

In this paper we report the results of calorimetric studies on the thermodynamic stability of two 24-mer DNA triplexes in which the TFO contains a 3'-3' internucleoside phosphodiester linkage and the target double helix is constituted by two palindromic strands. The two helices differ from the substitution of the cytosine (CC-triplex) with the thymine (CT-triplex).The sequences are shown below:
where the symbols • and × indicate the Watson-Crick and Hoogsteen hydrogen bonds, respectively. The arrow indicates the substitution point.

CT-triplex	CC-triplex
5' 5' 3'	5' 5' 3'
T×A•T	T×A•T
T×A•T	T×A•T
C×G•C	C×G•C
T×A•T	T×A•T
C×G•C	C×G•C
T×A•T	T×A•T
T×A•T	T×A•T
C×G•C	C×G•C
C×G•C	C×G•C
T×A•T	T×A•T
T×A•T	T×A•T
C×G•C	C×G•C
C•G×T ←	C•G×C ←
T•A×T	T•A×T
T•A×T	T•A×T
C•G×C	C•G×C
C•G×C	C•G×C
T•A×T	T•A×T
T•A×T	T•A×T
C•G×C	C•G×C
T•A×T	T•A×T
C•G×C	C•G×C
T•A×T	T•A×T
T•A×T	T•A×T
3' 5' 5'	3' 5' 5'

Many reports devoted to thermodynamic analysis of triple helices based on calorimetric technique have been reported in the last years [9–15] but no report has appeared on calorimetric study of triple helices containing TFOs with polarity inversion. The calorimetric data here reported provide useful information on thermodynamic stability in solution, near physiological conditions, of the above described triple helices.

Materials and methods

Oligodeoxyribonucleotide synthesis and purification

The 3',5'-oligodeoxyribonucleotide was synthesized using standard solid-phase phosphoramidite methods [16]. The third strand containing a 3'-3' phosphodiester junction have been conveniently syntesized through a solid phase procedure involving only 3'-phosphoramidite nucleosides, and starting from a modified support that links the first nucleotide through the base [17].

Triplex preparation

The triplexes were formed by mixing stoichiometric amounts of oligonucleotides in the appropriate buffer and heating the solution at 90°C for 5 min. The solution was slowly cooled to room temperature, then equilibrated for one day at 4°C before performing DSC experiments.

The buffer used was 140 mM KCl, 5 mM NaH_2PO_4, 5 mM $MgCl_2$. Potassium chloride (Sigma), monosodium phosphate (Sigma) and magnesium chloride (Carlo Erba) were used as obtained from commercial suppliers. Each of the solutions was adjusted to desired pH values with 1 M HCl. The pH of solutions was measured using a Radiometer pHmeter model PHM 93 at 25°C.

Scanning calorimetry

DSC analysis was performed on a second generation Setaram Micro-DSC at 0.5°C min^{-1} speed. The calorimetric unit was interfaced to an IBM PC computer for automatic data collection and analysis using the software previously described [18]. The apparent molar heat capacity curve was obtained by correcting each calorimetric curve for the instrument calibration curve and buffer-buffer scanning curve and dividing by scan rate and the number of moles. The performance of the instrument was calibrated periodically with an electrical pulse.

Results and discussion

pH dependence of thermal stability of CT-triplex

The thermal stability of the CT-triplex was studied on changing the pH from 6.0 to 7.0, keeping constant the concentration to $7.09 \cdot 10^{-5}$ M. The calorimetric data are collected in Table 1 and the calorimetric profiles are shown in Fig. 1.

The CT-triplex melts by two distinct processes whereas the isolated duplex melts by a single transition. In the selected conditions the thermal denaturation is highly

reversible, as demonstrated by the recovery of the original signal rescanning of the same sample. Furthermore, the change of the heating rate from 0.3 to 1°C min^{-1} does not alter the thermodynamic parameters significantly, thereby demonstrating that the studied processes are not kinetically limited.

Table 1 Thermodynamic parameters of the CT-triplex on changing the pH and keeping constant the concentration to $7.09 \cdot 10^{-5}$ M

pH	Process*	CT-triplex	
		T_m/°C**	$\Delta H(T_m)$/kJ mol^{-1}
6.0	I	44.4	298± 6
	II	74.3	721±12
6.2	I	38.8	310±10
	II	74.3	712±10
6.4	I	32.1	302±13
	II	74.4	730±21
6.6	I	25.2	320±10
	II	74.4	709±20
7.0	I	17.7	306±61
	II	74.4	712±18

* I Process: $A_2D \leftrightarrow A_2+B$
II Process: $A_2 \leftrightarrow 2A$
**The error in T_m does not exceed 0.2°C

The enthalpy change relative to the second transition of the triplex, $\Delta H(T_m)$=709±20 kJ mol^{-1} is in perfect agreement with the enthalpy change of 711±24 kJ mol^{-1} found for the transition of the isolated duplex.

For both the processes the van't Hoff enthalpies were calculated from calorimetric data according to the equation [19]:

$$\Delta H_{v.H.} = 6RT_m^2 \Delta C_p(T_m)/\Delta H(T_m) \quad (1)$$

where T_m is the maximum of DSC peak and corresponds to α=0.5, $\Delta C_p(T_m)$ is the value of the excess heat capacity function at T_m and $\Delta H(T_m)$ is the calorimetric enthalpy directly obtained by the area under DSC peak. Generally the closeness to unity of $\Delta H_{v.H.}/\Delta H(T_m)$ ratio states that a transition proceeds in a two-state manner. van't Hoff enthalpies are 304 and 710 kJ mol^{-1} for the first and second process, respectively. The ratio $\Delta H_{v.H.}/\Delta H(T_m)$ approaches to unity for both the transitions suggesting that each process can be considered as a two-state process [19] according to the schemes:

$A_2B \leftrightarrow A_2+B$ I process
$A_2 \leftrightarrow 2A$ II process

where A_2B indicates the triple helix, A_2 the target duplex and B the TFO.

The thermal stability of duplex is independent of pH, between pH 6.0 and 7.0, whereas the thermal stability of triplex is pH dependent. This is not a surprising re-

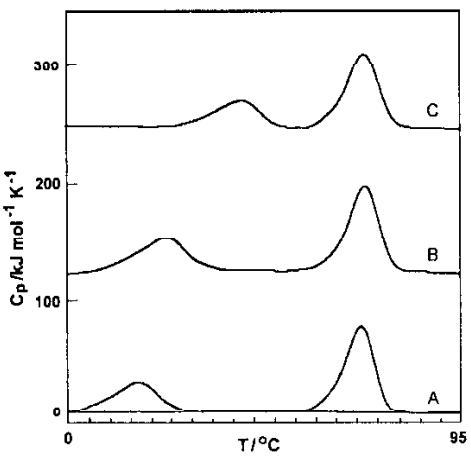

Fig. 1 DSC profiles of CT-triplex on changing the pH: (A) pH=7.0; (B) pH=6.6; (C) pH=6.0. Excess heat capacity values have been shifted along the y-axis for ease presentation

sult because it is consistent with the request that the cytosines on the TFO must be protonated.

Asensio and co-workers [20] showed by NMR studies that the pK_a of cytidine residues involved in Hoogsteen hydrogen bonds was about 9.5. Hence, in the pH range between 6.0 and 7.0, we can assume that all the cytosines in the triplex are protonated. However, upon the melting, the cytosine residues on TFO became partially protonated and the protonation degree is pH dependent. Consequently, the estimated calorimetric enthalpy comprises the contribution from the cytosine protonation. Manzini *et al.* [11] calculated the enthalpy of cytidine protonation and they found a value of –2.8 kcal mol^{-1} of cytidine. Furthermore, from their data we can calculate the fraction of protonated cytidine at pH 6.0 and 7.0. This fraction is about 2 and 0.2% at pH 6.0 and 7.0, respectively; so that considering that there are 9 cytosine residues on TFO, the protonation enthalpy is at the most: 0.02×9×(–2.8)= –0.504 kcal mol^{-1}=–2.1 kJ mol^{-1}.

This value is less than 1% of the estimated calorimetric enthalpy and it falls in the experimental uncertainty. Indeed, we found that the enthalpy for the triplex to duplex and TFO transition does not vary in the narrow range of explored pH. Because, on increasing the pH, T_m decreases and $\Delta H(T_m)$ remains constant, the pH dependence of CT-triplex thermodynamic stability is entropically controlled.

In order to calculate the denaturation entropy at each pH, being the denaturation process depending on stoichiometry, it is necessary to use the equations:

$$\Delta S = \Delta S^{\circ} - R\ln Q \tag{2a}$$

$$\Delta S^{\circ} = \Delta H(T_m)/T_m + R\ln Q \tag{2b}$$

In fact ΔS is the negative temperature derivative of the Gibbs energy :

$$\Delta G = \Delta G^\circ + RT\ln Q \tag{3}$$

where Q is the reaction quotient and in this case $Q=[A_2]\,[B]/[A_2B]$. Under the experimental condition selected the concentration is $7.09\cdot10^{-5}$ M, so that at $T=T_m$ (i.e., when the advancement degree of the process is 0.5) is equal to $3.54\cdot10^{-5}$ M. From Eq. (3) we can solve for ΔS and we obtain the values of 875 J mol^{-1} K^{-1} and 964 J mol^{-1} K^{-1} at pH 6.0 and 7.0 respectively.

In order to perform a correct thermodynamic comparison the enthalpy, entropy and Gibbs energy must be all referred to the same temperature. We found $\Delta C_p \approx 0$ for this process, as already found in calorimetric studies on triplex transition [15]. Consequently ΔH and ΔS do not vary with the temperature. Then it is possible to calculate ΔG at T=298 K and hence, from the well known thermodynamic relationship

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \tag{4}$$

we obtain:

ΔG° =305–298 (0.875)=44.2 kJ mol^{-1} at pH=6.0
ΔG°=305–298 (0.964)=17.7 kJ mol^{-1} at pH=7.0

These values reveal that the relative thermodynamic stability of CT-triplex ($\Delta\Delta G^\circ$=26.5 kJ mol^{-1}) is overwhelmingly entropic in origin, as we already pointed out, and derives from cytosine deprotonation upon triplex melting. Taking into account the release of the protons, the triplex transition, that gives the duplex plus the TFO, must be modified as follows:

$$A_2B(nH^+) \leftrightarrow A_2 + B + nH^+ \tag{5}$$

where n is higher at pH 7.0 than pH 6.0. Hence the entropy change of the triplex melting is greater at higher pHs than at lower pHs. Consequently, the inverse process

Table 2 Comparison between the thermodynamic parameters of CT-triplex and CC-triplex. C=4.17·10^{-5} M

pH	Process*	CT-triplex		CC-triplex	
		T_m/°C	$\Delta H(T_m)$/ kJ mol^{-1}	T_m/°C**	$\Delta H(T_m)$/ kJ mol^{-1}
6.0	I	42.2	310±10	43.6	330±11
	II	73.7	715±17	73.5	716±18
6.6	I	23.9	300±14	28.3	337±15
	II	73.6	704±15	73.4	706±19
7.0	I	16.4	312±45	19.3	321±44
	II	73.5	716±18	73.6	720±20

* I Process: $A_2B \leftrightarrow A_2+B$
II Process: $A_2 \leftrightarrow 2A$
**The error in T_m does not exceed 0.2°C

that carried out to triplex formation and stability is entropically favoured by lowering the pH.

Comparison between CC-triplex and CT-triplex stabilities

In order to compare the thermal stabilities of CT- and CC-triplexes the DSC measurements were performed in the same experimental conditions (Table 2).

The CC-triplex also melts by two distinct transitions (Fig. 2) and the second peak perfectly corresponds to that of duplex transition.

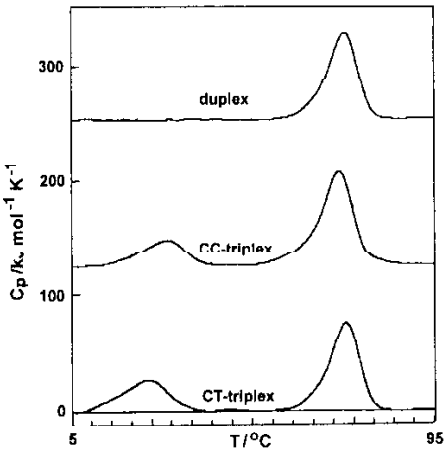

Fig. 2 DSC profiles of the two triplexes and the target duplex at pH=6.6. Excess heat capacity values have been shifted along the y-axis for ease presentation

The most streaking results in the comparison regards to the Process I are: i) the transition enthalpies of CC-triplex are slightly greater than CT-triplex and do not vary with the pH; ii) T_m of CC-triplex is greater than that of CT-triplex at each investigated pH. In order to make a correct comparison between the triplex stabilities, we calculated ΔG° values at 298 K and pH 6.6 using the Eqs (2) and (4). The calculations give a $\Delta\Delta G^\circ$ value of 4.8 kJ mol^{-1}, i.e. in terms of Gibbs energy the CC-triplex is 16% more stable than CT-triplex.

In conclusion, small differences in pH, near the neutrality, and the substitution of one base, in a critical point, involve relevant differences on triplex stability.

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