

# **Gas Phase Thermal Denaturation of an Oligonucleotide Duplex and its Complexes with Minor Groove Binders**

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Running title: GAS PHASE THERMAL DENATURATION OF DNA COMPLEXES

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## Abstract

Electrospray ionization with in-source CID has been used to probe the gas phase stability of an oligonucleotide duplex and its complexes with some minor groove binding drugs. On the basis of the arguments developed in detail by L. Drahos et al., *J. Mass Spectrom.* **34**, 1373 (1999), this type of experiment can also be described as "thermal denaturation in the gas phase". We found that these gas phase denaturation curves were very similar to the solution phase denaturation curves determined by the traditional UV spectrophotometric method and, by analogy with the melting temperature  $T_m$  characterizing the stability in solution, we define a melting voltage  $V_m$  characterizing the stability in the gas phase. The comparison between the  $T_m$  and  $V_m$  relative values suggests that the structure of the complexes is conserved during the electrospray process which transfers the ions from the solution to the gas phase.

## Introduction

The introduction of the electrospray ionization method<sup>1</sup> has permitted the mass spectrometric analysis of large biomolecules, including fragile non-covalent complexes<sup>2</sup>. Electrospray is often called a "soft" ionization method, because the analytes can be given little internal energy during their transfer to the gas phase, so that even fragile species having low fragmentation barriers can be observed intact by mass spectrometry.

According to the RRKM statistical theory<sup>3</sup>, the fragmentation rate depends on the activation barrier to be overcome, on the internal energy the ions are given, and on the degrees of freedom over which the internal energy can be redistributed. The internal energy of the ions leaving the source is the sum of the energy present before ionization (equal to their thermal energy if the ions are in the electronic ground state and at thermal equilibrium), the energy given during the ionization process, and the energy subsequently given on purpose by collisions with the ambient gas during the acceleration in the intermediate-pressure region of the source. The latter can be modulated by tuning the "acceleration voltage", which is the voltage applied on the skimmer separating the atmospheric pressure source from the low-pressure region of the mass analyzer. The internal energy distributions of probe ions produced by electrospray have been determined previously as a function of different experimental parameters<sup>4,5</sup>. The experimental energy distributions clearly resemble the Boltzmann type, so that a characteristic temperature can be attributed to each distribution. This characteristic temperature is defined as the temperature of the thermal energy distribution which would give the same parent/fragment ratio as the one observed<sup>5</sup>. A very interesting feature revealed by this study<sup>5</sup> is the linear relationship between the so-defined characteristic temperature and the applied acceleration voltage; the slope of that linear regression depends on the number of degrees of freedom (DOF) of the molecule under consideration (the proportionality factor is

not known) and on the spectrometer used<sup>5</sup>. This means that increasing the acceleration voltage (which is an easily tunable experimental parameter) is equivalent to increasing the temperature of the ion in the gas phase. The great advantage of this in-source CID method is that results obtained with different source geometries can be readily compared by a simple calibration of each source with probe molecules<sup>6</sup>.

The probe molecules used so far are relatively small (75 and 228 DOF for benzylpyridinium and leucine enkephalin ions, respectively), and their fragmentation patterns involve the breakage of covalent bonds. In this paper we report the study of a far larger system involving non-covalent interactions, an oligonucleotide 12-mer duplex and its complexes with different minor groove binders. These drugs can form non-covalent complexes with AT-rich sequences in the minor groove of the duplex involving hydrogen bonds with the bases of the two strands<sup>7</sup>, and their binding properties are partly responsible for some antitumor activity. The first observation of non-covalent duplex structures by mass spectrometry date back to 1993<sup>8</sup>, and their specificity has been discussed in 1995 by Ding and Anderegg<sup>9</sup>. In 1997, small model oligonucleotide duplexes were investigated by the BIRD method by Williams et al.<sup>10</sup>, who correlated the stability of the duplexes in the gas phase with the number of hydrogen bonds between the strands, thereby inferring that the solution structure is in that respect maintained in the gas phase. Non-covalent interactions between duplex DNA and small drug molecules have also been investigated by electrospray ionization mass spectrometry<sup>11,12</sup>.

The goal of this study was twofold. First, we wished to investigate the influence of the acceleration voltage, and thus of the internal temperature, on a larger system (over 2300 DOF) in the absence of solvent. The above-mentioned drug-DNA complexes appeared to be particularly suitable because one of the charge states (5-) has a stability compatible with the range of acceleration voltages (0 → -135 V) available on the instrument (a Finnigan LCQ),

while the lower charged duplex (4-) remains intact. Second, we wished to compare the relative stabilities obtained by "denaturation" of the complexes in the gas phase by in-source CID with the thermal denaturation curves obtained in solution by the traditional UV spectrophotometric method.

## **Experimental**

Oligonucleotide single strands were chosen purposely to be non self-complementary and of significantly different masses to allow unambiguous interpretation of the spectra. Single strand d(GGGGATATGGGG) will be referred to as **G** (monoisotopic mass = 3,804.67 Da) and the complementary strand d(CCCCATATCCCC) will be called **C** (monoisotopic mass = 3,484.62 Da). Oligonucleotides were purchased from Eurogentec (Sart-Tilman, Belgium), annealed in 100 mM ammonium acetate to form the duplex (**GC**) which was desalted with MICROCON3 (Amicon, Beverly, MA) filters. The drugs Hoechst 33258 (Acros, Geel, Belgium) and netropsin (Serva, Heidelberg, Germany) were dissolved in 0.1 M ammonium acetate for the stock solutions.

Mass spectrometric measurements were performed using a Finnigan LCQ ion trap instrument (ThermoQuest, Bremen, Germany). The spray voltage was set to -3.8 kV (negative ion mode), the heated capillary temperature was set to 190°C and the cone voltage (acceleration voltage) was varied from 0 to -135 V. Each spectrum was acquired for 50 scans. The duplex concentration was 10 µM in a 80/20 (100 mM aqueous NH<sub>4</sub>Ac/methanol) solution, and was injected at 1 µl/min. The drug concentrations were 15 µM, to obtain the maximum intensity for the 1:1 complex at 0 V acceleration voltage.

Thermal denaturations were performed using a Lambda 5 UV spectrophotometer (Perkin Elmer, Norwalk, CT, USA) interfaced with a PC XT for data acquisition. The quartz cells

were heated by a Julabo F25 heating circulator (Kutztown, PA, USA). These measurements were performed with the duplex and complex concentrations of about 2.5  $\mu\text{M}$ , in order to have an absorbance range between 0.2 and 0.8. The concentrations were carefully set to the same value for the different UV-melting experiments by diluting aliquots of a duplex stock solution with 100 mM  $\text{NH}_4\text{Ac}$ , and adding the relevant drug if a complex is to be analyzed, to obtain a total volume of 1.1 ml. By doing so, the relative  $T_m$  values directly indicate the relative stability of the analyzed species (otherwise the  $T_m$ 's would also depend on the concentration)<sup>13</sup>.

## Results and discussion

Full scan mass spectra of the duplex and complex solutions were recorded at different acceleration voltages, with all other experimental parameters kept constant. The intensities measured for the duplex and complexes are nearly 100% (full scale) at acceleration voltages < 20 V. This is a *sine qua non* condition for the following discussion to be of any value, i.e. our complexes remain intact when exiting the source at low acceleration voltage (low internal energy, or low characteristic temperature). Figure 1 shows that rising the acceleration voltage induces the breaking of the duplex species into its single strands, among which the available charges are shared. For each spectrum, the relative intensity of the  $(\text{GC})^{5-}$  species was calculated as a function of the total ion current resulting from the duplex (see caption to Fig. 1), and the percentages of relative intensity obtained this way are plotted as a function of the acceleration voltage in Figure 2. The nature and the number of fragments taken into account in the normalization do not influence the shape of the curve, but the more numerous the considered fragments, the smoother the curve.

The spectra of the complexes were treated the same way. One can only compare the stabilities of complexes of the same charge state because of the coulombic repulsion. The charge state 5- has been chosen because the intensity of these peaks varies from 100% to 0% when varying the acceleration voltage from 0 to -135 V. As was previously noted for MS/MS experiments on the same complexes<sup>12</sup>, the nature of the fragments differs with the drug (data not shown); this feature has been already discussed in terms of the number of hydrogen bonds formed with each strand for a typical ATAT sequence<sup>12,14</sup>. The ability to identify the fragments resulting from the denaturation of the complex is of course one of the great advantages of mass spectrometry, compared to spectrophotometric methods which can only detect the weighted average of the effect of all the species present on a macroscopic physical property.

The results obtained by mass spectrometry for the duplex<sup>5-</sup> and complex<sup>5-</sup> species are summarized in Figure 3a in such a way that the visual comparison with the solution thermal denaturation curves (fig. 3b) is made easier. The curves of Figures 3a and 3b are strikingly similar, although the nature of the experiments is fundamentally different. In solution, a temperature increase induces the denaturation of the duplex, which manifests itself in a hyperchromism measured by UV-spectrophotometry<sup>15</sup> (single strands have a higher extinction coefficient  $\epsilon$  than the duplex). Minor groove binders form hydrogen bonds with both strands and thus act as a supplementary bridge between the strands of the duplex; the more thermodynamically stable the complex, the higher the melting temperature  $T_m$ <sup>16</sup> (temperature of half-denaturation). For in-source CID experiments<sup>17</sup>, however, one does not observe a temperature-dependent equilibrium, but the effect of the internal energy of the ions on the unimolecular fragmentation rate of the ion. When comparing the fragmentation rates of ions that have the same internal energy and the same number of degrees of freedom, the difference in stability is due to the activation barrier to be overcome, so the higher the activation barrier,

the higher the value of  $V_m$  (voltage of half-fragmentation, defined by analogy with  $T_m$ ). The  $V_m$  and  $T_m$  values are reported in Table 1. On a fundamental point of view, our results strongly indicate that the linear relationship between the acceleration voltage and the temperature<sup>5</sup> of the ions still holds for molecules of over 2,000 degrees of freedom. If the internal energy of the ions can be characterized by a temperature<sup>5</sup>, the denaturation caused by capillary-skimmer dissociation can be described as "thermal", and curves obtained in Figure 3a can be called "gas phase thermal denaturation curves".

The similarity between the solution phase and the gas phase thermal denaturation curves suggests that the interactions responsible for the relative stability of the complexes is conserved during the electrospray process which transfers the ions from the solution to the gas phase. The forces that are involved in the molecular recognition in solution of the ATAT central sequence by the minor groove binders are electrostatic interactions, hydrogen bonding with both strands, and van der Waals interactions with the edges of the minor groove<sup>18</sup>. It is known from the crystallographic structures that netropsin forms tighter hydrogen bonds with a central ATAT sequence than does Hoechst 33258<sup>14</sup>, and this explains why the complex with netropsin is more stable than the complex with Hoechst 33258 in solution. Our ESI-MS measurements indicate that the stability scale is the same in the gas phase, and on this basis the hydrogen bonding interactions are thus believed to be conserved. This in turn implies that the structure of such complexes is conserved during the electrospray process, thus providing a reason why ESI-MS has been found so suitable for the determination of the stoichiometries<sup>11,12</sup> and relative binding affinities<sup>11c-d,12</sup> of oligonucleotide complexes with minor groove binders.

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## Table legend

**Table 1.** Denaturation temperatures ( $T_m$ ) and denaturation voltages ( $V_m$ ) for the duplex GC and the complexes with the minor groove binders.

## Figure legends

**Figure 1.** Spectra of a 10  $\mu$ M GC duplex solution recorded at 40 V (a) and 90 V (b) acceleration voltages.

**Figure 2.** Relative intensity of the duplex as a function of the acceleration voltage. Each point was obtained from the relevant spectrum (see fig. 1), where the relative intensity has been calculated as follows (the intensities  $I$  were defined as the peak heights):

$$\% \text{Rel.Int.} = \frac{I_{GC^{5-}}}{I_{GC^{5-}} + (I_{C^{3-}} + I_{C^{2-}})} \cdot 100\% .$$

**Figure 3. (a)** Gas phase thermal denaturation curves obtained by mass spectrometry for the duplex  $GC^{5-}$  ( ) and for the complexes  $(GC+Hoechst)^{5-}$  ( ) and  $(GC+Netropsin)^{5-}$  ( ). The y axis is scaled from 100 to 0 to make easier a visual comparison with part b. **(b)** Solution thermal denaturation curves of the three species (same conventions) by measuring the absorbance increase at 260 nm.

	<b>T<sub>m</sub> (°C)</b>	<b>V<sub>m</sub> (V)</b>
Duplex	48.2	68
Duplex + Hoechst 33258	58.5	89
Duplex + netropsin	60.5	99





