Sodium Controlled Selective Reactivity of Protonated Deoxy-Oligonucleotides in the Gas Phase

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Metastable fragmentation of the positively charged, hexameric oligonucleotides 5'-d(TTXYTT) (X and Y are dC, dG, or dA) and 5'-d(CTCGTT), 5'-d(TTCGTC) and 5'-d(CTCGTC) is studied after matrix assisted laser desorption/ionization (MALDI). The influence of the degree of sodiation, i.e., when the acidic protons are one by one exchanged against sodium ions, is systematically studied for the exchange of up to seven protons against sodium ions. Exchanging the acidic protons against sodium gradually quenches the backbone cleavage through the w and a-B channels, and quantitative quenching of these channels is generally achieved with the exchange of four protons against sodium ions. At the same time, the exchange of protons against sodium ions promotes the loss of a neutral, high proton affinity base. The formation of the w and a-B fragments is found to be highly dependent on the sequence of the central bases. A single mechanism consistent with these observations is proposed. In addition to the quenching of the classical w and a-B reaction channels, a drastic and abrupt *on/off-switching* of new reaction channels is observed as the degree of sodiation successively increases. These channels involve selective loss of the two central bases and the excision of a phosphodiester group and a sugar unit from the center of the oligonucleotides. Synchronously, the two terminal fragments recombine to form a tetramer containing the two terminal nucleosides from each end of the hexamer. Possible mechanism explaining these remarkable channels are discussed. (J Am Soc Mass Spectrom 2009, 20, 689–696) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

The need for new analytical methods and mass spectrometric techniques to shed light on the complex molecular structure and composition of biologically relevant molecules has drawn the attention of physicists and physical chemists more in the direction of gas-phase studies on molecules such as DNA, RNA, proteins, and peptides. The introduction of the electrospray ionization (ESI) and the matrix-assisted laser desorption ionization (MALDI) in the early 90s and the further development of these methods throughout the last decade have revolutionized the studies of biologically relevant molecules. Especially in the field of proteomics, these methods have proven to be very powerful both for the identification of known proteins and for the sequencing of unknown proteins and peptides. The metastable decay of preselected parent ions in MALDI has also proven to be very useful in proteomics [1–3], and is today a key technique in sequence analysis in proteomics. However, at the same time, the performance of these methods has not met expectations when it comes to the analyses of DNA segments. One of

the difficulties hampering the use of MALDI TOF-MS in DNA analyses is the metastable and prompt decay of these molecules in the MALDI process [4–11]. Furthermore, the strong affinity of oligonucleotides to exchange their numerous acidic protons against sodium complicates the sample preparation considerably and often sets limits to the achievable resolution [4, 5]. In the last decades, several studies have been conducted to attempt to unravel the mechanism behind the prompt and metastable decay of oligonucleotides in MALDI [5–7, 9–26]. The bulk of this work is reviewed by Wan and Gross in 2001 [13] and by Wu and McLuckey in 2004 [6]. Several different mechanisms have been suggested for the decay of negatively charged oligonucleotides studied, and the experimental data does not appear to support one single mechanism [9, 10, 12–15]. For the protonated oligonucleotides, on the other hand, the bulk of the studies conducted show that the fragmentation (with the exception of the loss of terminal nucleotides or nucleosides and the loss of water) is initiated by the protonation of a high proton affinity base [6, 11, 14, 24–26]. The base then leaves the oligonucleotide either protonated or as the neutral base, and the base loss is followed by a backbone cleavage. Studies have also been conducted on the fragmentation of oligonucleotides with a single metal ion attached [27–33], and Wang et al. [34, 35] studied the collision

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induce dissociation (CID) of doubly negatively charged oligonucleotides with all the acidic phosphodiester protons exchanged against sodium ions. In these experiments the metal adducts of the $[M - 2H]^{2-}$ ions were generated through electrospray ionization and the fragmentation upon collisional activation was studied with an ion trap.

Recently, we published a study on the metastable decay of the sodium adducts and the sodium free singly charged deprotonated hexameric nucleotides 5'-d(TTXYTT) (X and Y are dC, dG, or dA) when formed in the MALDI process [12]. In this study, we found that the backbone cleavage of the deprotonated oligonucleotides is gradually quenched with increasing degree of sodiation and that quantitative quenching is generally achieved when four of the acidic protons have been exchanged against sodium ions. The base loss, on the other hand, increases with increasing sodiation, hence as the backbone cleavage decreases. Furthermore, when the sixth proton was exchanged against sodium and beyond that point we observed a relative destabilization of the pyrimidine bases compared with the purine bases. Our observations are concordant with the CID work of Wang et al. [34], which shows considerably higher stability for the metal adducts compared with the native $[M - 2H]^{2-}$ ions and that the loss of pyrimidine bases becomes favorable compared with the purine base loss when the phosphodiester protons are exchanged against metal ions.

Here we have extended our study to the post source decay (PSD) of protonated hexameric nucleotides 5'-d(TTXYTT) (X and Y are dC, dG, or dA). We study systematically the influence of the degree of sodiation, i.e., when the acidic protons are one by one exchanged against sodium ions, and we show that the role of sodium is not less significant for the protonated oligonucleotides. Similar to the case of the deprotonated oligonucleotides, we observe quenching of the classical w and a-B channels with increasing degree of sodiation of the protonated oligonucleotides, and we find the branching ratios between these two channels are highly dependent on the sequence and nature of the two central bases. In addition to the w and a-B channels, we observe a drastic and abrupt *on/of-switching* of different reaction channels as the degree of sodium increases. The new channels, which to our knowledge have not been observed before, involve selective excision of the two central bases as well as a phosphodiester group and a sugar unit, presumably also from the center of the oligonucleotides. To account for the mass observed a synchronous recombination of the two terminal d(TT) fragments formed in the process is required. To verify that the fragments formed still contain the two terminal nucleosides, we also studied PSD of 5'-d(CTCGTT), 5'-d(TTCGTC), and 5'-d(CTCGTC). Here we discuss our findings within the framework of a single mechanism being responsible for both the w and a-B fragmentation observed, and we discuss possible

explanations for the appearance of the new channel observed and for its abrupt disappearance.

Experimental

The hexameric nucleotides 5'-d(TTXYTT), where X and Y are dC, dG, or dA were all synthesized from the phosphoramidites of the nucleotides with standard protecting groups (Glen Research, Sterling, VA, USA). The syntheses were carried out on a Biossets ASM 800 DNA synthesizer (Novosibirsk, Russia). The oligomers were purified using 20% denaturing polyacrylamide gel electrophoresis, reverse phase HPLC and Sephadex G-15 column (Amersham Biosciences, Piscataway, NJ, USA). The purified samples were dried on a Thermo-Savant ISSI110 Speedvac system (Waltham, MA, USA) and redissolved in 100 μ L sterile, purified water. The concentration was determined by UV absorption at 260 nm on 500 times dilute samples. For storage the samples were diluted to 0.1 mM solutions, separated into 10 μ L portions, and stored at -20°C until used. The hexameric nucleotides 5'-d(CTCGTT), 5'-d(TTCGTC), and 5'-d(CTCGTC) as well as a control sample of 5'-d(TTCGTT) were all purchased HPLC purified from TAG Copenhagen A/S (Copenhagen, Denmark) and used without further purification. The samples were delivered dry and were dissolved in purified water to make a 0.1 mM solution before use.

The MALDI post source decay (PSD) spectra of the sodiated and nonsodiated oligonucleotides were obtained on a REFLEX IV (Bruker Daltonics, Bremen, Germany), a reflectron type UV-MALDI-TOF with a 400 μ J/pulse N_2 -Laser operating at 10 Hz and 337 nm. All experiments were carried out in positive ion mode and for each oligonucleotide, the protonated ions containing at least 0–7 sodium atoms were one by one selectively gated into the field free linear flight tube. The desorption-laser power was kept about 10% above the detection threshold for each oligomer and the laser spot was moved manually over the sample during acquisition to average out sample heterogeneity. The gate width was ± 5 u in all experiments and the ion extraction was operated in pulsed delayed extraction mode with 400 ns delay time. The total acceleration voltage through the linear region was 25 kV resulting in about 30 μ s flight time. This is the time window within which we observe metastable decay. After the linear flight, the ions were decelerated and reaccelerated with a grid-less reflectron and detected on a double micro-channel plate detector. The reflectron voltage was stepped down in seven segments to assure for collection of all fragments. Individual segment are the sum of 500 shots, which were recorded by using the fragmentation analyses and structural TOF (FAST) method, within the instrumental control software FlexControl. The alignment of individual segments and the mass calibration spectra was carried out with the FlexAnalyses software also provided by the instrument manufacturer. Mass accuracy was verified by comparing the measured mass of the protonated

molecular ion and that of the w_2 fragments with the calculated masses.

Before fragmentation analyses of the oligonucleotides, 2 μL of the 0.1 mM sample solutions were mixed with 0.5 μL of 0.01 M NaCl solution to increase the degree of sodiation of the samples. From the sodiated samples 2.5 μL were then mixed with 2 μL of a saturated aqueous solution of 2,5-dihydroxybenzoic acid. The sample/matrix mixtures were spotted on a polished stainless steel sample carrier and allowed to dry in air.

Results and Discussion

Figure 1 shows PSD spectra of protonated 5'-d(TTCGTT) ions containing 0–7 sodium ions. Similar to the deprotonated hexameric oligonucleotides, which we reported in an earlier study [12], the predominating channels observed for the protonated hexameric oligonucleotides are backbone cleavage through the w_3 and the $a_4\text{-B}_4\text{H}$ fragmentation channels as well as single base loss. To a lesser extent we observe backbone cleavage through the w_2 channel. Like in the case of the deprotonated oligonucleotides the w and the $[a_4\text{-B}_4\text{H}]^+$ fragmentation channels are very effectively quenched in the protonated oligonucleotides as the degree of sodiation increases. These observations are qualitatively the same for all oligonucleotides studied, and generally quantitative quenching of the w^+ and the $[a_4\text{-B}_4\text{H}]^+$ fragment formation from the protonated oligonucleotides is achieved with the exchange of four protons against sodium ions in the parent ions. This is in good agreement with the increased stability of the oligonucleotides observed by Wang et al. [34] after exchanging all the acidic phosphodiester protons against metal ions. However, when comparing these experiments one should keep in mind that in their experiment they were study-

Figure 1. Post-source decay spectra of the protonated 5'-d(TTCGTT)-3' ion where 0–7 protons have been exchanged against sodium ions.

Figure 2. The ratio of the intensity of the w_3^+ and the $[a_4\text{-B}_4\text{H}]^+$ channels to that of the total backbone cleavage through both channels. The ratio is shown for all base and sequence combinations, whereby CG for example signifies that guanine is at the third position and cytosine at the fourth position of the base.

ing the sodium adducts of the $[M - 2H]^{2-}$ ions and here we are studying the sodium adducts of the $[M + H]^+$ ions. Contrary to the backbone cleavage we find that the base loss generally increases with increasing degree of sodiation. This is, however, only true for the exchange of up to five protons against sodium ions. In this range, we find the stability of the bases to be inversely related to their proton affinities (PA); $\text{PA}(\text{G}) = 959.5 \text{ kJ/mol}$, $\text{PA}(\text{C}) = 949.9 \text{ kJ/mol}$, $\text{PA}(\text{A}) = 942.8 \text{ kJ/mol}$, $\text{PA}(\text{T}) = 880.9 \text{ kJ/mol}$ [36]. Hence, we observe increasing single base loss in the order $\text{T} \ll \text{A} < \text{C} < \text{G}$ when up to five protons have been exchanged against sodium ions. This relationship between the stability of the oligonucleotides and the proton affinities is also apparent in the relative intensity of the w_3^+ fragments compared with the $[a_4\text{-B}_4\text{H}]^+$ fragments. In the case of 5'-d(TTGCTT) and 5'-d(TTCGTT), the w_3^+ channel is more prominent than the $[a_4\text{-B}_4\text{H}]^+$ channel when G is in the third position and the $[a_4\text{-B}_4\text{H}]^+$ fragment is more intense when G is in the fourth position. In the case of the oligonucleotides 5'-d(TTGATT) and 5'-d(TTAGTT) this relationship is more apparent. The $[a_4\text{-B}_4\text{H}]^+$ channel dominates when G is in the fourth position and the w_3^+ channel when G is in the third position. The situation is similar for the oligonucleotides 5'-d(TTCATT) and 5'-d(TTACTT), but now in favor of the loss of neutral cytosine. Figure 2 shows this relationship between the w_3^+ and the $[a_4\text{-B}_4\text{H}]^+$ channels as a fraction of the total backbone cleavage through both channels. It is apparent from Figure 2 that the higher the proton affinity of the base in the fourth position the higher is the intensity of the $[a_4\text{-B}_4\text{H}]^+$ fragment compared with the sum of both the w_3^+ and the $[a_4\text{-B}_4\text{H}]^+$ fragments. To the same extent it is clear from Figure 2 that the higher the proton affinities of the base in third position, the more preferable becomes the w_3 channel. This is concordant with the initial step being the protonation of a high proton affinity base leading to a weakening of the glycosidic bond and the loss of the neutral base. A 1',2'-elimination can then be

Scheme 1. Proposed mechanism for the formation of w_3^+ from 5'-d(TTGCTT) as representative for the 5'-d(TTXYTT) oligonucleotides studied. For clarity, only the free electron pairs and the carbon bound hydrogens that are directly involved in the mechanism are shown.

completed through a proton transfer from the 2'-carbon of the sugar moiety to a neighboring high proton affinity base. This initial step was previously suggested by Gross et al. for the formation of w_2 fragments from positively charged tetrameric nucleotides [14]. In this case (formation of w^+ fragments) the proton transfer takes place through the 3'-phosphodiester group. In Scheme 1 a proposed mechanism is shown for 5'-d(TTGCTT) where the highest proton affinity base is in the third position, hence w_3^+ is the fragment preferably formed. In this scheme the initial base elimination step is followed by a 3',4'-elimination through a rupture of the 3'-phosphodiester bond and a transfer of the 4'-hydrogen to the phosphate group. The charge remains on the 3'-fragment and thus the resulting charged fragment is w_3^+ . In Scheme 2 a similar mechanism is proposed for 5'-d(TTCGTT) in which the highest proton affinity base is in the fourth position. In this case $[a_4-B_4H]^+$ is the preferably formed fragment. The initial step is the same as proposed for the w_3^+ fragment formation in Scheme 1, i.e., the loss of the neutral guanine, induced through its initial protonation. Like in the mechanism shown in Scheme 1 the 1,2-elimination initiated by the base loss is completed by a proton transfer from the 2'-carbon of the sugar moiety to the next neighboring base with the highest proton affinity. The difference is that in this case it is at the 5'-end of the sugar moiety and the proton transfer takes place through the 5'-phosphodiester group. Consequently,

the following 3',4'-elimination leads to charge retention on the 5'-end and thus the formation of $[a_4-B_4H]^+$. In short, if the initially protonated base is at the third position of the 5'-d(TTXYTT) nucleotides the charge remains preferably on the 3'-fragment as shown in Scheme 1 leading to the formation of a w_3^+ . If, on the other hand, the initially protonated base is at the fourth position of the 5'-d(TTXYTT) nucleotides, the charge remains preferably on the 5'-fragment resulting in the formation of a $[a_4-B_4H]^+$.

In our experiment, we observe that the backbone cleavage via the w_3^+ and the $[a_4-B_4H]^+$ channels comes to a halt when the acidic phosphodiester protons are exchanged against sodium, and that the intensity of the signal from the single base loss (relative to that of the parent ion) increases with increasing degree of sodiation. This is concordant with a mechanism where the base loss is independent of the availability of acidic phosphodiester protons, but the backbone cleavage is critically dependent on these. Hence the fragmentation is bound to come to a halt after the base loss if no protons are available at the adjacent phosphodiester groups. Furthermore, this mechanism also explains our observation that the branching ratio of the w_3^+ and $[a_4-B_4H]^+$ fragmentation channels is highly dependent on the nature and sequence of the central bases. However, when the sixth proton is exchanged against sodium also the purine base loss is efficiently quenched but at the same time the loss of the pyrimidine bases

Scheme 2. Proposed mechanism for the formation of $[a_4-B_4H]^+$ from 5'-d(TTCGTT) as representative for the 5'-d(TTXYTT) oligonucleotides studied. For clarity, only the free electron pairs and the carbon bound hydrogens that are directly involved in the mechanism are shown.

cytosine and thymine is facilitated. This is most apparent in the case of 5'-d(TTGCTT) and 5'-d(TTCGTT) (see Figure 1). As mentioned above, this is the same observation we made for the negatively charged oligonucleotides $[M - H]^-$ [12] and Wang et al. made earlier for the doubly deprotonated $[M - 2H]^{2-}$ ions generated in their electrospray experiments [34]. This is somewhat surprising as it indicates that partly the same mechanisms are operative in the dissociation processes of these different ions.

However, as the quenching of the w_3^+ and the $[a_4-B_4H]^+$ fragment formation progresses, we observe two new channels that open up. The more prominent first appears at 1376 u for the 5'-d(TTCGTT) parent ion when two protons have been exchanged against sodium ions (see Figure 1). The intensity of the peak, which is shifted by 22 u for each additional proton exchanged against sodium, progressively increases until the parent ion contains five sodium atoms. When the sixth proton is exchanged against a sodium ion, this channel abruptly closes as if it was *switched off*. The same observation is made for all the oligonucleotides studied and, more importantly, the mass to charge ratio observed for this fragment is the same for all the oligonucleotides, independent of the nature and sequence of the central bases.

This is shown in Figure 3 where the fragmentation pattern of all six oligonucleotides studied are compared when; (a) five and (b) six protons have been exchanged against sodium ions. It can be seen that the sixth sodium abruptly switches off this channel independent of the nature or the sequence of the two center bases. It is also clear from Figure 3 that the mass to charge ratio of the ions formed through this new reaction channel are the same for all the oligonucleotides. Therefore, clearly the fragment

Figure 3. Comparison of the fragmentation pattern of all six 5'-d(TTXYTT) oligonucleotides when; (a) five and (b) six protons have been exchanged against sodium ions.

Figure 4. Comparison of the fragmentation pattern of 5'-d(TTCGTT), 5'-d(CTCGTT), 5'-d(TTCGTC), and 5'-d(CTCGTC) in the mass range from 1300 to 1500 u when five protons have been exchanged against sodium ions (left side). The mass selected parent ions $[M + 5Na - 4H]^+$ are shown on the right side.

formed does not contain the two central bases. A closer look at the mass to charge ratio also shows that the ions have not only lost the two central bases but also one sugar and one diphosphoester unit. These must come from the center of the backbone as the ionic fragment still contains both the terminal thymidine groups, d(TT). Consequently the process comprises the excision of the central bases, a phosphodiester group and a sugar unit, and the synchronous recombination of the two terminal d(TT) groups. To verify that this fragment is indeed composed of the two terminal bases from both the 5' and the 3' end of these oligonucleotides we carried out additional PSD experiments on 5'-d(CTCGTT), 5'-d(TTCGTC), and 5'-d(CTCGTC) as well as control experiments on 5'-d(TTCGTT) from the same supplier. Figure 4 compares the fragmentation pattern of 5'-d(TTCGTT), 5'-d(CTCGTT), 5'-d(TTCGTC), and 5'-d(CTCGTC) in the mass range from 1300 to 1500 u when five protons have been exchanged against sodium ions (left side). For comparison, the mass selected molecular ions $[M + 5Na - 4H]^+$ are shown on the right side. In all cases we observe the same fragmentation pattern as for the other oligonucleotides 5'-d(TTXYTT), except that the mass is now shifted by 15 mass units for 5'-d(CTCGTT) and 5'-d(TTCGTC) and by 30 mass units for 5'-d(CTCGTC), i.e., shifted by the mass difference between thymine (126 u) and cytosine (111 u). This shows clearly that the fragment in question is composed of the two terminal nucleosides from each end of the oligonucleotides studied.

From the strong dependence on the degree of sodiation, we conclude that this reaction takes place from a structure that is most favorable when the oligonucleotides contain five sodium ions, which coincides with the

Scheme 3. Proposed mechanism for the excision of two nucleobases, a sugar unit and a diphosphoester unit from the center of the hexameric oligonucleotides with synchronous recombination of the terminal d(TT) groups.

number of the acidic phosphodiester protons available. However, this structure is also attainable with as few as two protons exchanged against sodium ions. With the sixth sodium ion on the parent ion this structure can not be attained and the reaction channel is turned off, or the sixth sodium ion blocks one of the reaction centers involved with the same consequences.

Scheme 3 shows a proposed mechanism for this reaction in which the reaction takes place over a cyclic phosphate intermediate leading to the formation of the protonated sequence (I) or the protonated sequence (II). The first sequence results from a recombination of the 5' end with the fourth phosphodiester group (marked A in Scheme 3) the second sequence results from a recombination

of the 3' end with the second phosphodiester group (marked B in Scheme 3).

Due to the rigidity of the 3'-OH-group we favor a transition-state involving the 5'-OH, i.e., reaction path (A). In this case the 5'-OH-group makes a nucleophilic attack on the phosphorous of the fourth phosphodiester group whereby the sodium of the leaving phosphate group (third position) must be transferred to the resulting terminal hydroxyl group on the sugar unit which is initially in position three. The leaving fragments are the two bases shown as cytosine and guanine in Scheme 3, the third phosphodiester group and the sugar moiety in fourth position. As indicated by the color scheme the resulting 3'-3' ion (I) contains the third sugar of the

parent ion in a terminal position. In this scheme the exchange of the proton on the 5'-hydroxyl group of the parent ion against the sixth sodium ion would bring the reaction to a halt. However, the difference of the sodium affinity (and the acidity) of the 5' primary alcohol and the secondary alcohol at the 3' position is not large. Thus, the exchange of a proton from one of the two terminal alcohol groups is not likely to be sufficiently selective to make such an abrupt closing of this reaction channel plausible. An exchange of a N – H proton at one of the central bases might be a more likely explanation for the reaction to come to such a sudden halt when the sixth proton is exchanged against sodium. This is plausible when the nucleophilic attack occurs after the base loss in which case the positively charged sugar moiety facilitates the reaction. The sixth sodium would in this case prevent the base cleavage from the oligomer and thus stop the reaction.

Conclusions

We observe a remarkable effect of the degree of sodiation on the unimolecular reactivity of oligonucleotides in the gaseous phase. The number of sodium ions exchanged against protons in the parent ions control with high selectivity the gas-phase reactivity of these ions. With increasing number of the acidic phosphodiester protons exchanged against sodium, the backbone cleavage through the w and a-B channels is gradually quenched, and quantitative quenching of these channels is generally achieved when four of the acidic protons have been exchanged against sodium ions. The neutral base loss on the other hand increases with increasing number of protons being exchanged against sodium ions. We explain these observations through a mechanism in which the initial protonation of the highest proton affinity base leads to the loss of the neutral base. A 1',2'-elimination is then followed by a 3',4'-elimination. As both elimination reactions proceed through a proton transfer mediated by a neighboring phosphodiester group these come to a halt when the phosphodiester protons are not more available. Consistent with our observations this mechanism leads primarily to the formation of w_3^+ fragments when the highest proton affinity base is at the third position of the hexameric oligonucleotides studied and predominantly to the formation of $[a_4-B_4H]^+$ when the highest proton affinity base is at the fourth position.

At the same time as the w_3^+ and $[a_4-B_4H]^+$ fragmentation channels are quenched, two new channels open up. The dominant channel comprises the excision of the two central bases, the central phosphodiester group and a sugar unit and also requires the synchronous recombination of the separating terminal groups. This channel gradually increases in intensity for parent ions containing 2–5 sodium ions and is abruptly *turned off* when the parent ion contains six sodium ions. We explain this sodium induced extraordinary selectivity by the hypotheses that the reaction takes place from a structure

that is most favorable when the oligonucleotides contain five sodium ions, but is also attainable with as few as two sodium ions. The sixth sodium on the parent ion either destroys the attained stereo-chemical prerequisites or, more likely, blocks a reaction center involved. This reaction center may be the primary reaction center predicted to be at the 5' OH group. However, it is more plausible that the sixth sodium exchanges a proton at one of the two central bases thus blocking the initial base elimination that facilitates the proceeding nucleophilic attack. This is consistent with a mechanism where the reaction proceeds through a cyclic phosphate intermediate initiated by a nucleophilic attack of a terminal OH-group on the centered phosphodiester unit.

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