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Comparison of electron capture dissociation and collisionally activated dissociation of polycations of peptide nucleic acids

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Electron capture dissociation (ECD) in Fourier transform ion cyclotron resonance mass spectrometry coupled with electrospray ionization enhances the sequence elucidation of peptide nucleic acids compared with conventional low-energy collisionally activated dissociation (CAD). Examples are shown where ECD produced complete or extensive sequence coverage in PNAs six to ten nucleobases long. However, facile base losses from the reduced species and low abundances of backbone ECD fragments presented a significant problem. This was rationalized through the lower degree of charge solvation on the backbone compared to polypeptides. Combination of both CAD and ECD data is advantageous, as these techniques produce cleavages at different sites. Copyright © 2001 John Wiley & Sons, Ltd.

Peptide nucleic acids (PNAs) have been used for the development of antisense and antigen drugs as well as for hybridization probes and tools in genetic diagnostics and molecular biology.[1–4] PNAs are DNA-mimics in which the deoxyribose phosphate backbone has been replaced by a polyamide backbone consisting of *N*-(2-aminoethyl)glycine units. The standard nucleotide bases are attached to the PNA backbone by a methylene carbonyl linker (Fig. 1). The binding of PNA to complementary DNA and RNA molecules obeys the Watson-Crick base pairing rules with formation of double helical complexes,[4] and, in general, PNA-DNA and PNA-RNA duplexes are more stable than the corresponding DNA-DNA, DNA-RNA and RNA-RNA duplexes. This is in part because the PNA backbone is uncharged and thus the duplexes with PNA lack electrostatic repulsion between the two strands.

In mass spectrometry (MS), electrospray ionization (ESI) is the most suitable ionization method for PNAs, since these molecules easily generate multiply charged ions[5] due to the high proton affinity of the nucleotide bases. Although a number of MS-based studies have been reported for identification and determination of base sequences of PNAs,[6–8] the problem remains largely unsolved. The main difficulty is that conventional tandem mass spectrometry (MS/MS) techniques employing collisionally activated dissociation (CAD) produce insufficient structural information on PNA molecular ions. The dominant fragmentation channels are not the backbone cleavages, as in CAD of peptides, but instead a water loss from the C-terminal primary amide and the cleavage of the methylene carbonyl linker.[7] The latter leads to elimination of a single nucleobase, with A and G losses being the most prominent.[8]

Electron capture dissociation (ECD) in Fourier transform ion cyclotron resonance mass spectrometry (FT-ICRMS) is a gas-phase reaction of multiply protonated molecules, MH_n^{n+}, with thermal (<0.2 eV) electrons.[9] Exothermic capture leads to the formation of a reduced radical ion, $MH_n^{(n-1)+}$, which, in the case of peptides, rapidly dissociates via the backbone N-C_α cleavage producing series of $\boldsymbol{c}$ and $\boldsymbol{z}\cdot$ fragment ions. The $\boldsymbol{c}$ and $\boldsymbol{z}\cdot$ series in ECD are normally more complete than the corresponding $\boldsymbol{b}$ and $\boldsymbol{y}$ series produced by CAD.[10] As a result of the non-ergodic nature of the ECD process (bond cleavage faster than randomization of the recombination energy), strong backbone bonds are cleaved even in the presence of much weaker bonds.[9,11] In the present work, we investigate the performance of ECD versus CAD for structural analysis of PNAs using as parent ions the electrospray-produced multiply charged species.

EXPERIMENTAL

Materials

The PNAs, a 6-mer (H-CAGCAG-NH_2), a 8-mer (H-TCGTCGAT-Lys-NH_2), and a 10-mer (H-ATGTCAGTCA-NH_2), used in this study were synthesized and purified as described.[19]

Electrospray ionization used 10 μM solutions of PNAs in water/methanol/acetic acid (49:49:2 v/v/v). All water used was obtained from a Milli-Q system (Millipore, Bedford,

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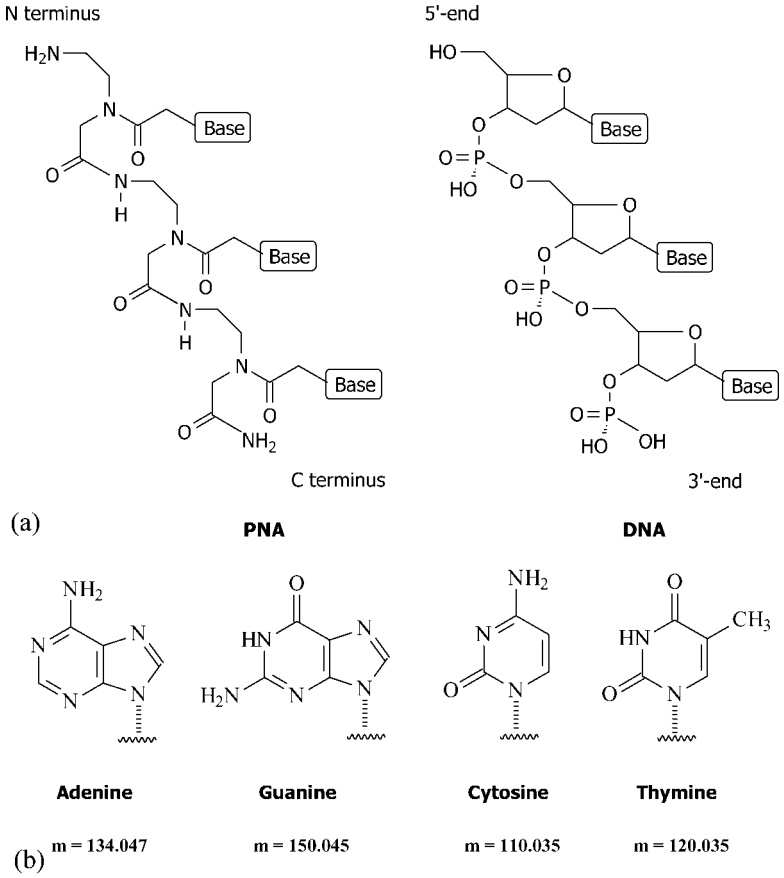

Figure 1. (a) Peptide nucleic acids (PNA) are analogues of deoxyribose nucleic acids (DNA). The sugar-phosphate backbone in DNA is replaced by a pseudo-peptide backbone in PNAs. (b) Common nucleobases that are also PNA bases.

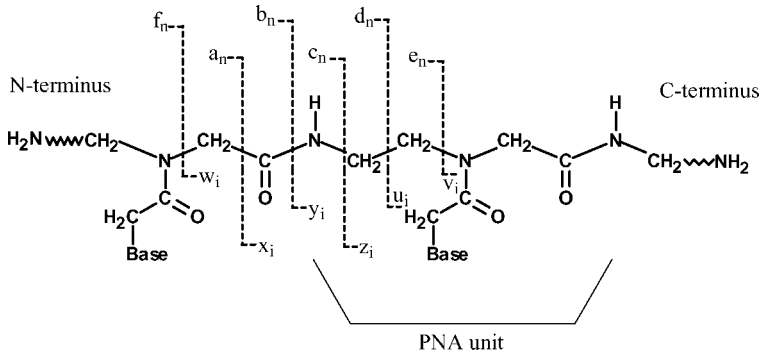

Figure 2. PNA fragmentation nomenclature used in this study.

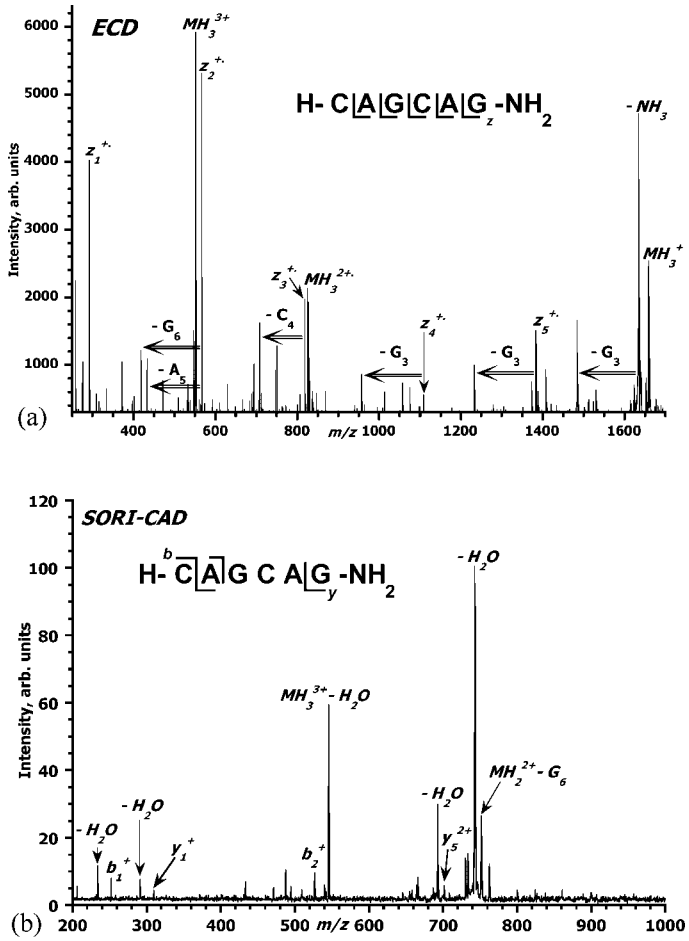

Figure 3. (a) ECD and (b) CAD spectra of the triply protonated peptide nucleic acid H-CAGCAG-NH_2 (M_r 1651.672, *m/z* 551).

MA, USA). All other chemicals used were ACS or HPLC grade.

Mass spectrometry

All experiments were performed using an Ultima Fourier transform mass spectrometer (IonSpec, Irvine, CA, USA) equipped with a 4.7 Tesla superconducting magnet, a custom-built nano-electrospray ion source and a standard hexapole-based electrospray interface (Analytica of Branford, MA, USA). Data acquisition and analysis were conducted using the IonSpec Omega data station and IonSpec software. For nano-electrospray MS analysis, a 3-μL aliquot of sample solution was loaded into the nano-electrospray needle (precoated borosilicate capillaries; MDS Proteomics, Odense, Denmark). The ions were externally accumulated in the rf-only hexapole for 500 ms, and then transported to the cell where they were captured in a gated trapping mode without a cooling gas. The precursor ions for MS/MS were isolated by on-resonance ejection of undesired ions. In the ECD experiments, precursor ions were irradiated for 8 s with subthermal electrons (<0.2 eV) produced by a standard tungsten filament installed behind the FT cell and biased to +1.25 V. At the end of the irradiation event, a 2-ms pulse of a cooling gas (nitrogen) at 20 Torr pressure was applied to remove an excess of the kinetic and internal energy from the fragment ions. Alternatively, the ions were collisionally activated by an off-resonance excitation (SORI CAD)[12] after a 4-ms nitrogen gas pulse. The total length of a single experiment was 10 s in both dissociation techniques, which allowed for accumulation of multiple scans within a several-minute run.

RESULTS AND DISCUSSION

PNA fragmentation nomenclature

Muddiman *et al.*[8] and previously Takao *et al.*[5] have suggested novel PNA fragment nomenclatures. For practical convenience, here we use more traditional backbone fragmentation nomenclature (Fig. 2). It is based on the commonly accepted notation[13] for peptides, close relatives of PNAs in terms of the backbone. In this nomenclature, C(O)-NH bond cleavage in a PNA containing x nucleobases gives $\boldsymbol{b}_n$ and $\boldsymbol{y}_i$ fragments ($n + i = x$), while N-CH_2 cleavage in ECD yields $\boldsymbol{c}_n$ and $\boldsymbol{z}_i$ products. Fragmentation of the CH_2-C(O) bond gives $\boldsymbol{a}_n$ and $\boldsymbol{x}_i$ fragments, similar to the fragments arising from the C_α-C(O) bond cleavage in peptides. The analogues of the ***a, b, c, x, y*** and ***z*** fragments are the **b, c, d, v, w** and **x** fragments in Muddiman's nomenclature.

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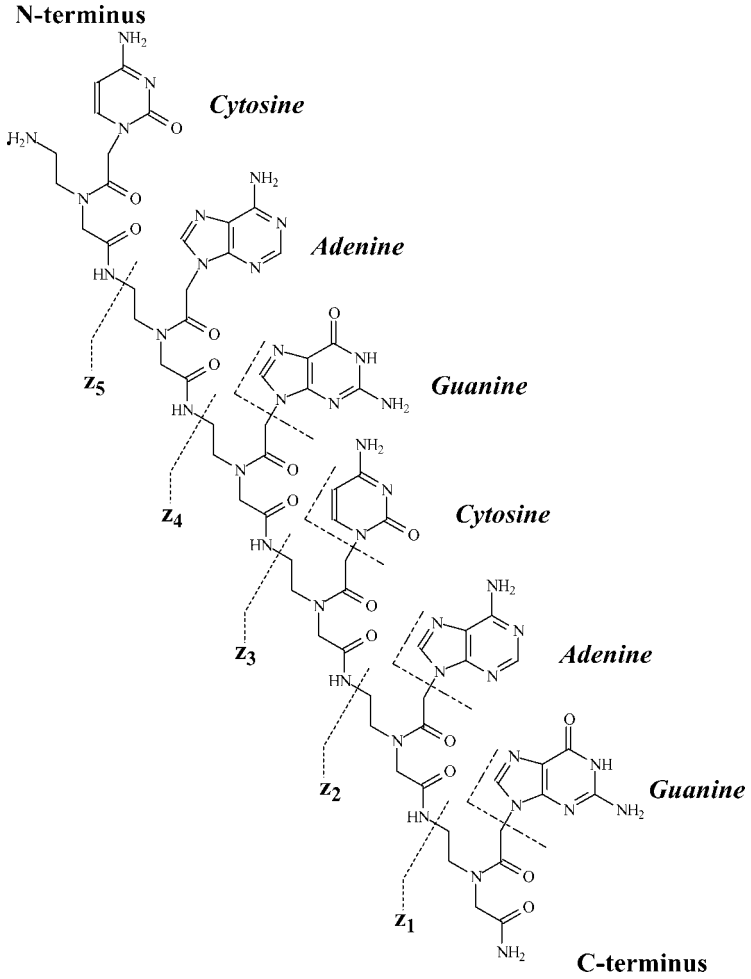

Scheme 1. ECD fragmentations in the 6-mer peptide nucleic acid molecule.

Comparison of ECD and CAD MS/MS spectra of PNAs

6-mer PNA

The ECD spectrum of 3+ ions of the 6-mer PNA H-CAGCAG-NH_2 (*m/z* 551) shown in Fig. 3(a) contains a complete series of singly charged $z^{\cdot}$ ions, from which the full sequence can be readily deduced. Other abundant fragment ions are due to the loss of a nucleobase from $z^{\cdot}$ ions. Especially prominent is the loss of mass 150, corresponding to guanines (G_3 and G_6). The main ECD fragmentation channels of this PNA are shown in Scheme 1.

For SORI-CAD of the 6-mer PNA (Fig. 3(b)), facile losses of water from the molecular ions were observed, consistent with the observations and calculations made by Flora *et al.*[6,8] Sequence ions from three out of five possible backbone cleavages are present in the CAD spectra; these are the b_1^+, b_2^+, y_1^+, and y_5^{2+} ions. The base peak in the spectra is due to the loss of a protonated guanine base and one water molecule.

8-mer PNA

The ECD spectrum of quadruply protonated hybrid PNA H-TCGTCGAT-Lys-NH_2 (*m/z* 577) is shown in Fig. 4(a). The most abundant peaks were due to non-dissociative electron capture, while the sequence-specific ions were of low abundances. Nevertheless, five out of the total eight inter-residue N-CH_2 bonds were cleaved, with four c and two $z^{\cdot}$ ions present, composing one complementary pair. The corresponding SORI-CAD spectrum (Fig. 4(b)) gave only three backbone cleavages. The CAD fragments were predominantly triply charged ions, b_7^{3+}, b_8^{3+} and y_8^{3+}. This spectrum also displays characteristic neutral losses of water from both the parent ions and the sequence-specific fragment ions.

10-mer PNA

The ECD spectrum of quadruply protonated 10-mer PNA H-ATGTCAGTCA-NH_2 (*m/z 682*) (Fig. 5(a)) also displays low-abundance backbone fragments compared to the stable reduced species. Five c ions and one $z^{\cdot}$ ion were present. In total, six out of nine backbone bonds were cleaved generating nearly complete nucleobase sequence coverage. The combination of G followed by T in the sequence was particularly stable, as no fragmentation between the two G-T bonds was observed for this molecule. The C and G base losses from the reduced parent ions gave the dominant peaks in the spectrum. The CAD spectrum of 5+ of the same 10-mer PNA again exhibited fewer backbone cleavages (four) than the ECD spectrum. The b_1, b_7 and b_8 ions were observed,

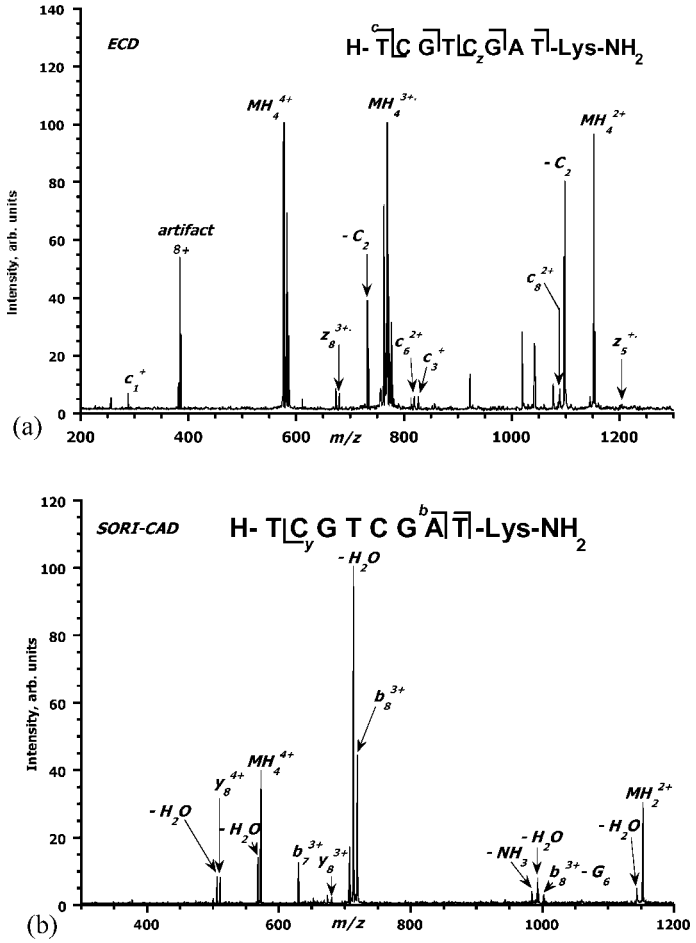

Figure 4. (a) ECD and (b) CAD spectra of 4+ of the 8-mer hybrid peptide nucleic acid H-TCGTCGAT-Lys-NH_2 (M_r 2302.953, *m/z* 577).

as well as the y_9 ion. Remarkably, one of the most intense peaks in the spectrum was due to the a_9 ion. Combination of the two fragmentation methods was sufficient to determine the nucleobase composition and nearly complete sequence, with the order within one (G + T) pair remaining uncertain.

Comparison of ECD of polypeptides and PNAs

Generally, polypeptides of comparable sizes and charge states produce in ECD far more abundant backbone cleavages than PNAs gave in this study. The most likely explanation is in the differences in the sites of protonation and charge solvation between these two classes of biopolymers. Facile nucleobase losses in PNA upon electron capture are in stark contrast to the preservation of labile groups in ECD of polypeptides.[14–17] These losses can be due to protonation of the nucleobases, the sites with high proton affinities.[6] The protons located on the nucleobases are likely to be shared with the neighboring nucleobases rather than the backbone carbonyls due to steric reasons. The proton sharing with the backbone carbonyl is considered crucial for backbone ECD cleavage;[18] the low probability of such sharing means low cleavage frequency. Besides, PNAs contain two carbonyl groups per attached nucleobase, which leads to competition between them. According to Muddiman *et al.*, the backbone carbonyl has ca. 15 kcal/mol higher proton affinity than that of the methylene carbonyl linker.[6] One can expect that higher charge states would contain labile protons that could protonate the backbone carbonyl. However, for the PNAs used in this study, attempts to achieve abundant backbone fragmentation using higher charge states have so far been unsuccessful.

CONCLUSIONS

In this study, ECD and SORI-CAD were used to characterize sequences of three small PNA oligomers. The dominant fragments in ECD are ***c*** and ***z*** ions in our nomenclature, similar to the ECD peptide fragments. Continuing the analogy with peptides, CAD produced ***b*** and ***y*** ions, as well as facile water losses. The ***c***, ***z***· fragmentation in PNAs is far less abundant than in polypeptides. This is explained by the lower degree of charge solvation on the backbone and the presence in PNAs of more than one carbonyl group per nucleobase. Despite this, ECD clearly gave a larger amount of structural information, in one case sufficient for full sequence identification. The losses of nucleobases have only been observed from radical ***z***· ions, consistent with the previously reported[16] larger stability of the even-electron ***c*** ions. Complete mass spectrometric sequence elucidation of PNA oligomers could not be achieved by one fragmentation

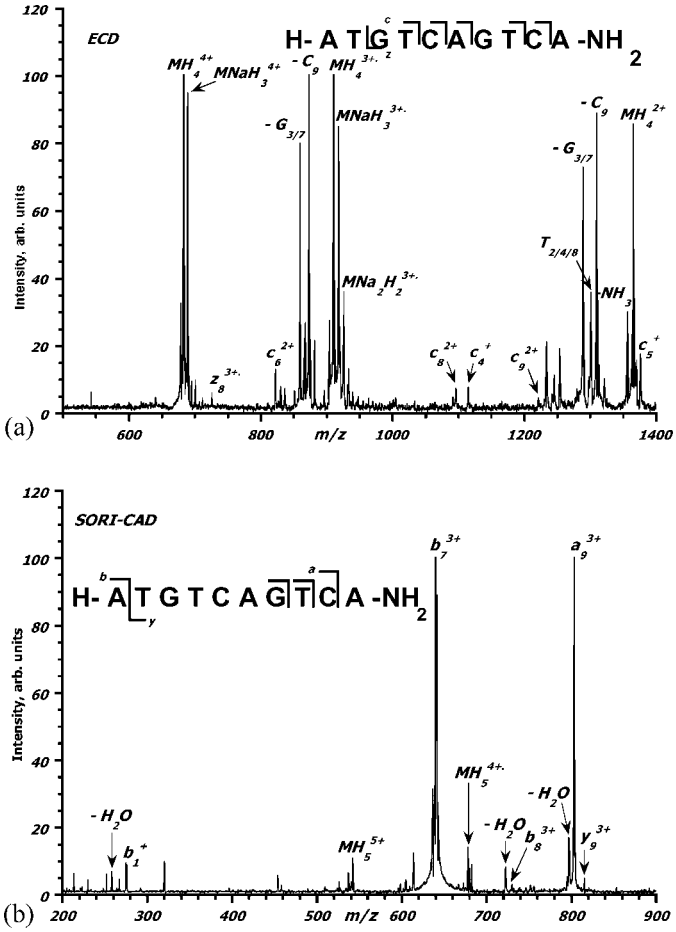

Figure 5. (a) ECD of 4+ of the 10-mer peptide nucleic acid H-ATGTCAGTCA-NH_2 (M_r 2726.0475, *m/z* 682) and (b) CAD of 5+ of the same molecule.

technique, but a combination of both ECD and CAD may provide the necessary structure information.

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