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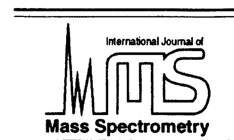
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Accelerated Communications

Hydrogen/deuterium exchange of monomers and dimers of leucine enkephalin

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Abstract

Leucine enkephalin has been studied using the combination of electrospray ionization (ESI) with a fast flow technique. ESI of leucine enkephalin produces an isotopic multiplet of peaks beginning at *m/z* 556. Hydrogen/deuterium exchange of this multiplet with ND_3 has revealed the contribution of two ion populations to this multiplet: The singly protonated monomer and the doubly protonated dimer. These populations were separated through their different kinetic behavior. Whereas the dimers undergo slow exchange the monomers undergo pronounced complexation with ND_3 and display a fast exchange of four labile hydrogens. The results indicate a more compact globular structure for the diprotonated dimer. (Int J Mass Spectrom 213 (2002) 1–4) © 2002 Elsevier Science B.V.

Keywords: Dimers; Electrospray ionization; Fast flow; H/D exchange; Leucine enkephalin

1. Introduction

Electrospray ionization (ESI) has been known [1] to produce multiply protonated peptides and proteins. These occur at different *m/z* ratios than the singly protonated species and are easily distinguishable. A fascinating discovery has been [2] the occurrence of doubly protonated dimers of leucine enkephalin at the same *m/z* ratio as the corresponding singly protonated monomers. These were uncovered through solvation by water and alkanols.

We have recently combined [3] ESI with a fast flow technique and have studied complexation and hydrogen/deuterium (H/D) exchange reactions of a series of protonated peptides with ND_3 and CH_3OD under thermal conditions. Among other peptides we have also studied the protonated pentapeptide leucine enkephalin, Tyr-Gly-Gly-Phe-Leu [4]. Deconvolution of the experimental mass spectral data followed by simulation of the kinetic data by solution of differential equations lead to sets of apparent and site-specific rate constants. On a time scale of several millisec-

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onds, leucine enkephalin undergoes with ND_3 four fast H/D exchanges and one slow exchange. Evidence was presented for the presence of two noninterconverting ion populations with different reactivities however, the source of these two populations was not uniquely identified [4].

The present letter is a short report on the discovery in the reaction system, of the doubly protonated dimer of leucine enkephalin, through its reduced reactivity in the H/D exchange reaction with ND_3 compared to the singly protonated monomer.

2. Experimental

The ESI/fast flow apparatus has been described in detail before [3,4]. It consists of a selected ion flow tube apparatus that we have constructed several years ago and modified to work with an electrospray source connected directly to the flow tube. It consists of a flow reactor that is 123 cm in length and an inner diameter of 74 mm. A neutral reagent is introduced into the flow tube through either one of two ring inlets. Tylan mass flow controllers define the flow rate of the neutral reactant into the flow tube. The quadrupole mass analyzer (652601 ABB EXTREL) is housed in a differentially pumped chamber that is separated from the flow tube by a nose cone (NC) skimmer with a 1.0 mm sampling orifice. A small NC voltage is used for focusing ions into the analysis quadrupole. Helium buffer gas enters the flow tube at the upstream end through another Tylan flow controller. A Roots blower pumps it through the tube. Typical flow tube pressures ranging from 0.2 to 0.3 Torr and reaction times of several ms were employed.

The electrospray ion source was designed as follows. A capillary tube serves as the interface between the electrospray and the helium flow reactor. Stainless steel tubes 15 cm in length and 0.05 cm in diameter are employed. The entire assembly is inserted into the flow tube at a distance of ~96 cm from the sampling orifice, 135° to the direction of the helium flow, through an 'O'-ring type vacuum fitting. A capillary tube of 0.05 cm in diameter introduces an air leak into the flow tube with a pressure of 0.07 Torr and a flow rate of 1.3 l/min (standard temperature and pressure). Ions are electrosprayed ~10 mm through ambient air into the grounded capillary tube from a stainless steel syringe needle biased at 5 kV DC. Dilute solutions of the analyte of interest in a polar solvent are delivered to the electrospray needle at flow rates of 3.3 μL min^{-1} from a 5000 μL syringe mounted on a model 100 KD Scientific Syringe Pump. The temperature of the capillary tube as well as of the flow tube is in the range of 22–30 °C.

Leucine enkephalin was purchased from Sigma/Aldrich (St. Louis, Missouri), with a stated minimum purity of 98%. A $\sim 10^{-4}$ M solution of the peptide was prepared in a 49:50:1 water-methanol-acetic acid mixture for introduction into the ESI source. ND_3 and CH_3OD were from Sigma/Aldrich with a stated isotopic purity of ≥99 at. %D.

3. Results and discussion

The elemental formula of protonated leucine enkephalin $(LeuEnk+H^+)^+$ is $C_{28}H_{38}N_5O_7$. This corresponds to an ion at a mass to charge ratio of *m/z* 556. Fig. 1, top panel, reproduces the isotopic multiplet obtained for electrosprayed leucine enkephalin (LeuEnk) arriving at the detector without introduction of any neutral reagent into the flow tube. If there is any contribution of a doubly protonated dimer, $(LeuEnk+H^+)_2^{2+}$ it would also appear at *m/z* 556. At the mass resolution available for the quadrupole mass filter employed, it is nearly impossible to detect within the experimental peak multiplet any contribution of the dimer. However, closer scrutiny of the peak shape suggests a hint of an ion at *m/z* 556.5 due to ^{13}C and/or other isotopic contributions to the dimer peak. Upon introduction of ND_3 into the flow tube the mass spectrum, Fig. 1 bottom panel, separates out into two groups of peaks. There is a group of four or even five peaks separated by half mass units at *m/z* 556, 556.5, 557, 557.5, and possibly also 558, respectively. There is a second group of three peaks separated by single mass units at *m/z* 559, 560, and 561, respectively. (The peak at *m/z* 558 may be the first member of this second group of peaks). The strongest peak

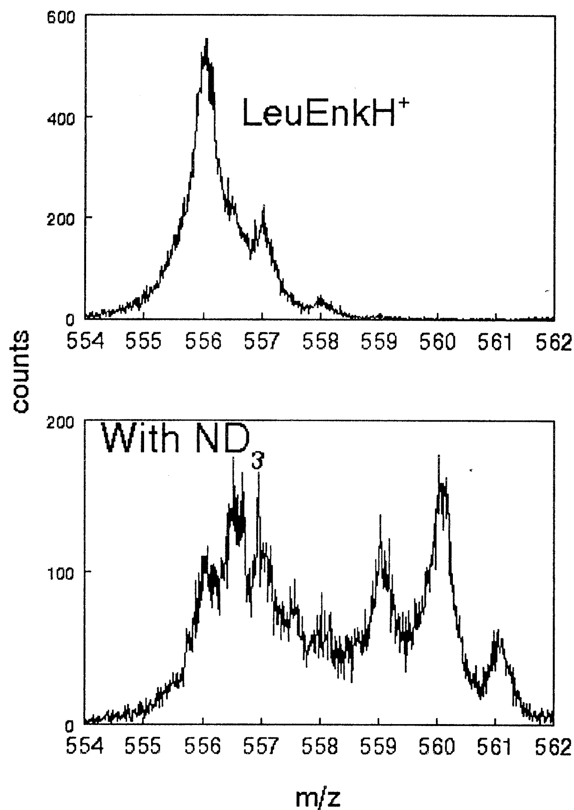

Fig. 1. Mass spectra of protonated and deuterated monomers and dimers of leucine enkephalin (LeuEnk, M = 556) without (top panel) and with (bottom panel) the H/D exchanging reagent, ND_3, flowing through the flow tube. Helium flow: 2.8 l/min; pressure: 0.21 Torr; Temp.: 295 K; ND_3 flow 8×10^{17} molecules/s.

among the group separated by single mass units is at *m/z* 560 and corresponds as before [4,5] to the rapid incorporation of four deuterium atoms into the singly protonated monomer of leucine enkephalin. The group of peaks separated by half mass units is ascribed to partially deuterated doubly protonated dimers of leucine enkephalin. The two groups of peaks get separated upon deuteration. If the doubly protonated dimer were demonstrating the same rapid incorporation of four deuterium atoms as the singly protonated monomer, then the multiplet of doubly protonated ions would peak at *m/z* 558. However, the strongest peak among the group of peaks separated by half mass units is at *m/z* 556.5. This constitutes evidence in favor of a slower incorporation of deuterium atoms into the dimer than into the monomer.

Conformational changes in proteins are probed by hydrogen/deuterium-exchange electrospray-ionization mass spectrometry [6,7]. The generally held idea has been that compact structures protect some labile hydrogen atoms from H/D exchange in the gas phase. As in the case of liquid solutions the reduction in amide hydrogen exchange rates in proteins in the gas phase is attributed to certain hydrogens such as those buried in the hydrophobic core, having little access to the solvent, or hydrogens participating in hydrogen bonding such as those found in α-helices or β-sheets [8]. As a result, open conformers are expected to reach higher levels of exchange than compact ones.

The experimental result reported here is interpreted as indicating a more compact structure for the doubly protonated dimer of leucine enkephalin than for the singly protonated monomer. For the same reaction time there are one or two fast H/D exchanges in the dimer per three to four exchanges in the monomer. Conformational structures of biopolimers in the gas phase can be probed by the technique of ion chromatography (ion mobility) [9]. It has been demonstrated [10] that aggregates of peptides that occur at the same mass-to-charge ratio as the singly charged parent ions are separable through their ion mobility distributions. Furthermore, collision cross sections for these clusters show that they have tightly packed roughly spherical conformations. Diprotonated dimers have been demonstrated to have shorter arrival times than the corresponding monomers, demonstrating again a more compact structure [11].

The present preliminary results for H/D exchange kinetics are thus in agreement with ion mobility studies. What is the exact structure of the diprotonated dimer remains an open question. With increasing ND_3 flow rates the dimer is observed to undergo further exchange. Furthermore, both the monomer and the dimer undergo complexation with ND_3 (Fig. 2). The degree of complexation increases with carrier gas flow rate. The ease of complexation of the monomer is as expected for an open conformer that undergoes quite efficient H/D exchange [3d,4]. The observation of complexation of the dimer with ammonia is ex-

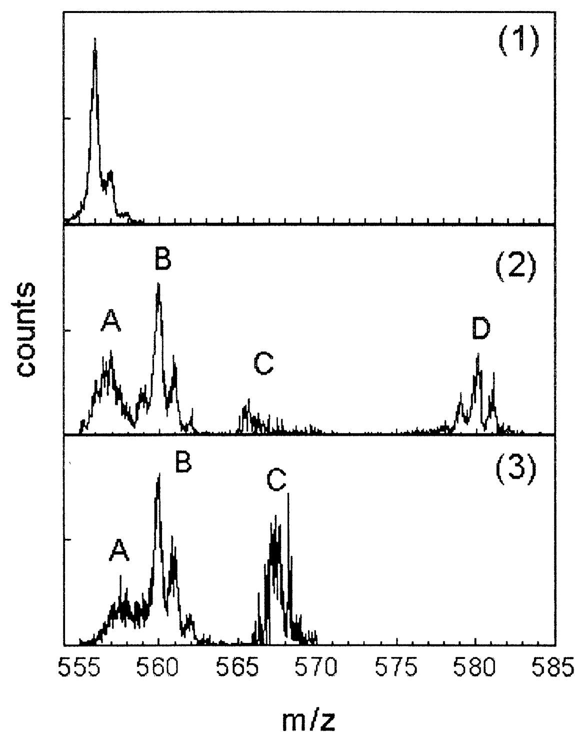

Fig. 2. Mass spectra on an expanded mass scale. (1) Unexchanged LeuEnk isotopic multiplet; He flow 3.7 l/min.; pressure 0.26 Torr; 295 K; (2) with ND_3 flow $= 6.1 \times 10^{17}$ molecules/s; (A) Partially resolved, partially deuterated isotopic multiplet of the diprotonated dimer; the intensity maximum is at $\sim m/z$ 556.5; (B) Partially deuterated isotopic multiplet of the singly protonated monomer; (C) Unresolved ND_3 complex of the dimer multiplet; the intensity maximum is at m/z 565.7; (D) The ND_3 complex of the partially deuterated isotopic multiplet of the singly protonated monomer. The multiplets at (B) and (D) are shifted by exactly 20 mass units (ND_3, $M = 20$) from one another. (3) Same as in (2) but with an ND_3 flow of 1.34×10^{18} molecules/s; the multiplet at (D) was not remeasured; the intensity maximum at (A) is at m/z 557.5 and at (C) at m/z 567.5, i.e. the peaks are shifted by $m/z = 10$ as expected for an addition of ND_3 to a doubly charged ion at (A).

pected on the basis of the previous solvation studies with water and alkanols [2]. The more compact dimer that undergoes less efficient H/D exchange than the monomer is still able to undergo complexation with ammonia and the complex is collisionally stabilized under the flow tube conditions. This is contrary to the results that we have obtained for the very compact diprotonated monomeric bradykinin ion for which no complexation with ND_3 was observable in the flow tube experiment [3d].

Acknowledgements

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