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Mass spectrometry of proteins—Uppsala perspectives on past and present☆

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Abstract

The development of biological mass spectrometry has been rapid in the past three to four decades. In particular, the possibility to detect and identify peptides and proteins from biologically and medically relevant samples has revolutionized life sciences. The development has gone from a stage where the detection of insulin in a mass spectrum was a major event to one in which the recording of mass spectra with more than 10^4 resolved and calibrating peaks in each spectrum is a routine task.

In this paper, the evolution of protein mass spectrometry will be discussed from the Uppsala horizon with special emphasis on the unique coupling between ion induced desorption of biomolecules and ion track physics.

Keywords: Mass spectrometry; Plasma desorption; Liquid chromatography; Fourier transform; Protein

1. Introduction

Mass spectrometry was born little over 100 years ago when the famous Nobel prize winner Sir J.J. Thompson identified a number of low mass ions in his parabola mass spectrograph [1]. Despite this respectful age, the field of mass spectrometry is more alive than ever before. In modern society, mass spectrometry is used to identify illegal drugs used by athletes, in forensic investigations, in space science, in the analyses of environmental pollutants, in diagnostics of disease, to determine the age of archaeological artifacts and in high sensitivity elemental analysis, just to mention a few examples [2].

The field that has become most prominent in recent years is protein identification by mass spectrometry. This is due in large part to the spectacular sequencing of the entire Human Genome, the HUGO project [3], as well as genomes of other species. The challenge for the scientists today is to identify all the proteins coded for in these genomes, determine the extent of their posttranslation modification and to determine their function in the host organisms. In this Sisyphean task, modern mass spectrometry has turned out to be indispensable.

Many factors have contributed to the exponential development of protein mass spectrometry over the last four decades. Techniques have been introduced that produce gas-phase ions of proteins by bombarding thin protein layers with ions or photons, with or without a matrix, and by electrospraying protein solutions. New mass analyzers have been introduced that employ time-of-flight with pulsed extraction and electrostatic mirrors, different kind of ion traps, and multipoles. Particularly successful have been hybrid instruments, that combine time-of-flight and quadrupole analyzers or integrate ion trap and Fourier transform ion cyclotron resonance analyzers. Other factors contributing to the fast development are the enormous advances in both computer hardware and software. Today, large amounts of data can be collected, analyzed, and processed with a conventional PC. Availability of software packages and databases accessible over the Internet has considerably reduced the time

☆ Paper in honor of Prof. Peter Roepstorff's 65th birthday.

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consumed in analyzing data. Equally important for the rapid growth of mass spectrometry has been the increase in number of companies marketing instruments. The mass spectrometers then were bulky, complicated, and complex in design and manufacturing. Today, several companies exist, and majority of the instruments are smaller and less complicated to manufacture. The total time from the inception of an idea to a ready product is surprisingly short!

There can be no doubt that in regard to proteins and other biological molecules, the nearly simultaneous introduction of MALDI [4] and electrospray [5] was a breakthrough in the development of mass spectrometry. However, the first step in this direction was taken by Macfarlane and co-workers [6] when they introduced the plasma desorption mass spectrometry (PDMS). They discovered that fission fragments from a radioactive source, ^{252}Cf, could be used to desorb and ionize biomolecules deposited on a metallic backing. This phenomenon was immediately used as an ion source in a time-of-flight mass spectrometer. The PDMS technique was picked up by several groups, one being the Uppsala Ion Physics group headed by Bo Sundqvist.

From its beginning, the research in Uppsala followed two main directions. The first was to investigate the possibilities and limitations of the new PDMS method from an applications point of view. The second was to understand the desorption mechanism. These two research directions will be described in Sections 2 and 3, respectively. In Sections 4 and 5, the development of modern protein mass spectrometry and a new method for obtaining information about protein structure in solution using tandem mass spectrometry data will be described.

2. The early days

To be able to study the plasma desorption process, a time-of-flight mass spectrometer was built, see Fig. 1, and connected to a beamline from the Uppsala Tandem Accelerator. This machine produced a high-intensity, well focused beam of ions,

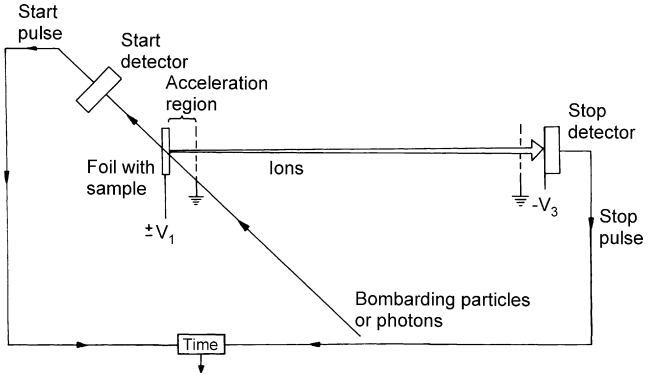

Fig. 1. The principle of a desorption/ionization time-of-flight mass spectrometer. The sample molecules are deposited on a metallic backing held at an acceleration potential. The bombarding particles can be fission fragments from a ^{252}Cf source (PDMS), fast heavy ions of MeV energy, slow heavy ions of keV energy (SIMS), atoms of keV energy (FAB), cluster ions, UV photons (MALDI) or IR photons (IR-MALDI). By measuring the time the desorbed ions need to travel a certain distance, their mass to charge ratio can be obtained.

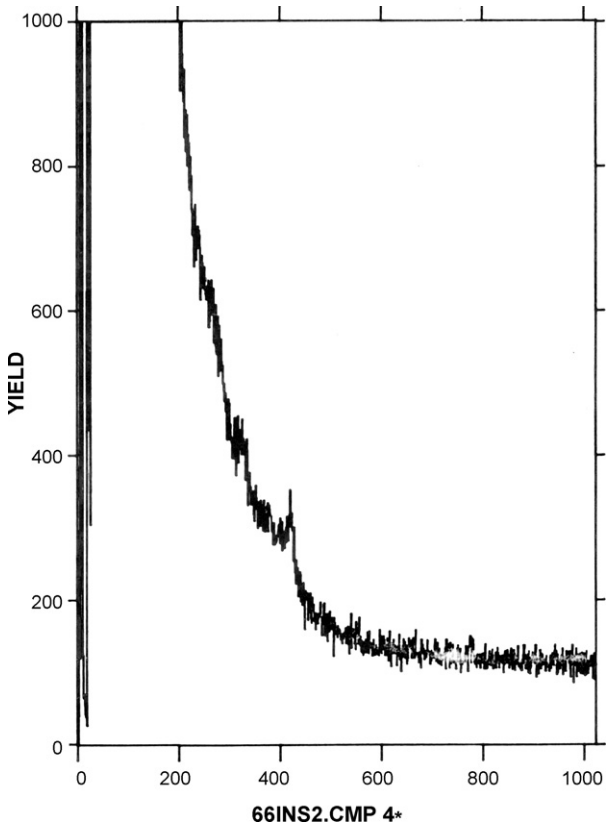

Fig. 2. Raw data from one of the first time-of-flight spectra from a sample of bovine insulin electrosprayed onto a probe and then desorbed by fast heavy ions using the Uppsala tandem accelerator.

with the same mass/energy of those produced with a ^{252}Cf source. Furthermore, the tandem accelerator produced ions with a fixed and selectable energy and mass that made them more suitable for mechanistic studies than ions from a fission fragment source that has a distribution of energies and masses.

During a visit by Macfarlane and McNeal to Uppsala in 1981, it was attempted to analyze bovine insulin. The samples were prepared by electrospraying a saturated solution of the insulin molecules in aqueous TFA onto a thin metallic backing. Surprisingly, a clear but low-intensity peak was observed on a steep decaying background, see Fig. 2. After improving the spectrum quality, both positive and negative ions were detected as well as the a and b chains of insulin, and the spectra were published in 1982 [7].

In the beginning of 1980s a collaboration was established between the Odense group, led by Peter Roepstorff, and the Uppsala Ion Physics group. This alliance turned out to be very successful and resulted in about 25 joint publications. Following the success with bovine insulin, a long line of different peptides and proteins were tested using the Uppsala mass spectrometer. All of these samples were provided by Novo in Copenhagen, Denmark, a company with which Peter Roepstorff had very good relations.

Despite the fact that small proteins and other difficult molecules could be detected by PDMS, the technique was

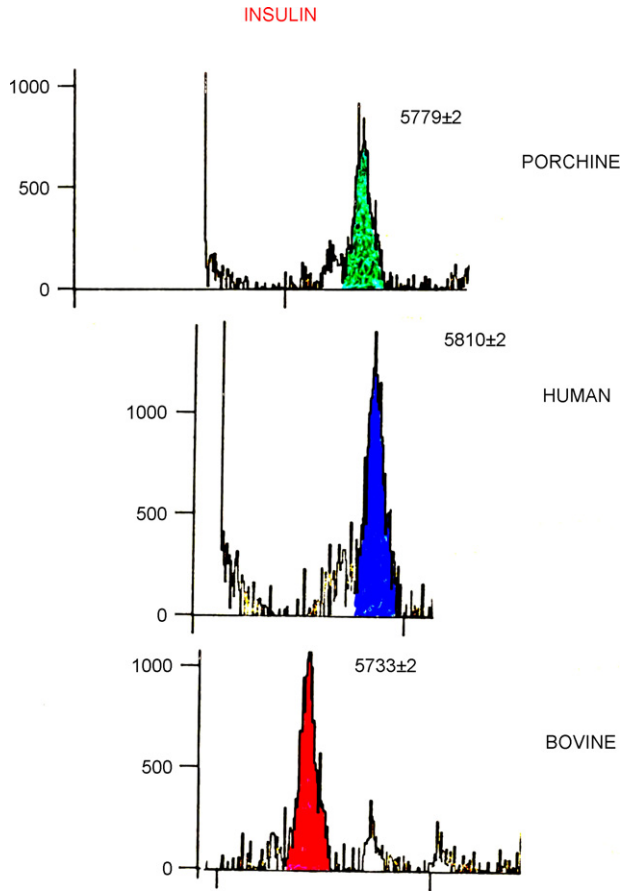

Fig. 3. Examples of protein mass spectrometry 25 years ago. Background subtracted spectra around the molecular ion region of positive ions from three different types of insulin, namely pig, human, and cow, respectively.

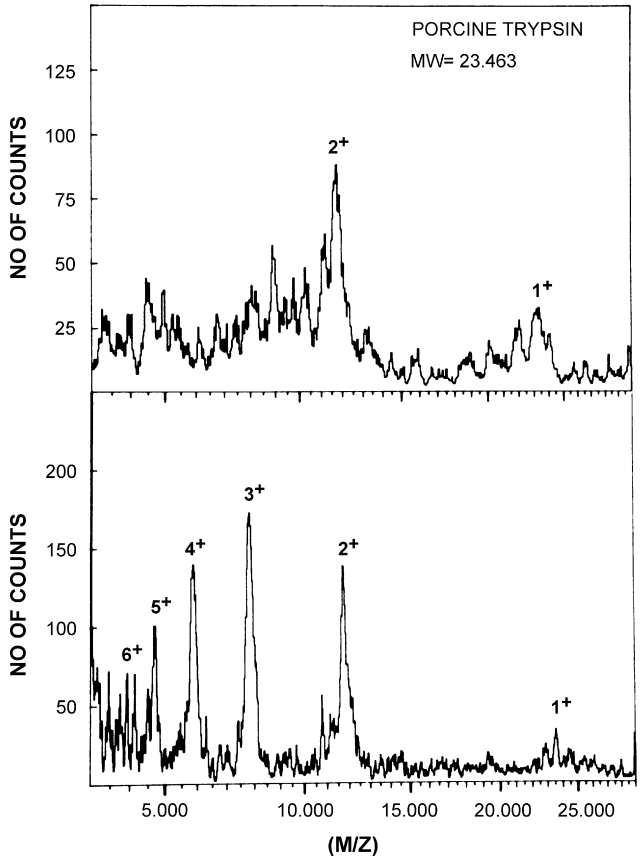

Fig. 4. Spectra from porcine trypsin showing the high-mass region for an electrosprayed sample, top, and a sample on a nitrocellulose film, bottom.

criticized because the mass resolving power was too low to be of any analytical interest. This objection notwithstanding, the centroid of a PDMS signal could be well defined even for a rather broad mass distribution, and it was demonstrated that different types of insulin could be distinguished, see Fig. 3, and later that the synthesis of human insulin could be monitored [8].

The best mass resolving power obtained for bovine insulin, desorbed by fast heavy ions, was close to 1800, which corresponds to the half-width of the envelope of the isotopic distribution. The spectrum was recorded using a time-of-flight spectrometer with an electrostatic mirror and a heavy ion beam focused to a diameter of 1 mm [9].

In 1984, 16 different peptides and proteins had been analyzed ranging in mass from LHRH (MW 1182) to phospholipase A2 (MW 13,980). The largest protein ions analyzed with the PDMS technique were singly charged molecular ions from a sample of ovalbumin (MW 45,000) adsorbed on a nitrocellulose (NC) backing [10].

2.1. Nitrocellulose

In 1986, a project was started with the goal to improve the detection of proteins separated on a gel and subsequently blotted onto a standard nitrocellulose membrane. The idea was to cut out the proteins on the NC membrane and analyze them with a PDMS spectrometer. The concept of loading samples of papers into the spectrometer was a "crazy experiment" that turned out to be a great success. It was found that the charging of the sample surface could be handled by spraying a thin layer of nitrocellulose on top of a metallic backing. If now a protein solution was applied to the NC film, salts and other contaminants could be rinsed away once the proteins had adsorbed to the NC film. The improvement in signal intensity was dramatic, see Fig. 4. Another surprising feature of the NC technique was that multiply charged ions were produced [11]. Multiply charged ions have a higher energy compared to singly charged ones and are therefore, more easily detected. Nitrocellulose, or "the magic Uppsala carpet" according to Frank Field, was an early example of a matrix playing an important part in the desorption/ionization process.

2.2. The Bio-Ion company

It became clear quite early that the PDMS technique was a unique tool for studying biomolecules. In 1983, a small company called Bio-Ion Nordic AB, in Uppsala, Sweden, was started by Hans Bennich with the goal to develop a commercial ^{252}Cf time-of-flight mass spectrometer. The executive director was Ivan Kamensky, who came out of the Ion Physics group together with Johan Kjellberg, Johan Blomberg and Maria Lindberg. The Bio-

Fig. 5. The proud personnel behind the development and production of the Bio-Ion Bin-10K instrument. From left to right: Ove Johansson, electronic engineer; Johan Blomberg, mechanics; Johan Kjellberg, research engineer; Torbjörn Söderström, mechanics; Mona Gustavsson, secretary; Maria Lindberg, chemistry; Ivan Kamensky, executive manager.

Ion spectrometer, see Fig. 5, was one of the first commercially available time-of-flight mass spectrometers on the market since the Bendix instrument disappeared in the 1960s. The first Bio-Ion Bin-10K instrument was delivered to Peter Roepstorff 1984; by 1990, Peter had received another Bin-10K and a Bin-20K spectrometer equipped with an electrostatic mirror for improved mass resolving power. These instruments were used until about 1997 by Peter Roepstorff's group.

In 1989, Bio-Ion was sold to Applied Biosystems and the company started to develop a MALDI instrument. However, Applied Biosystems merged together with Perkin-Elmer and the development of new instruments was stopped 1993. Bio-Ion lived until 1999 performing services on its running instruments. In retrospect, it is almost unbelievable that this small, almost garage type workshop, could manage to design, develop, produce and sell close to 50 ^{252}Cf time-of-flight mass spectrometers!

3. Electronic sputtering from bioorganic solids

3.1. The sputtering process

When an energetic particle hits a solid surface it causes material to be ejected from the surface, a process called sputtering. In the case of particles with velocities below the Bohr velocity, i.e., typical velocities of atomic electrons, the process was extensively studied and became an established research field. The ejection of atoms from the surface is a result of momentum transfer directly from the energetic ions to atoms of the solid. For fast ions in the so-called electronic stopping regime, the incoming ion interacts mainly with the electrons in the solid. For solids with slow electronic relaxation, like insulators, this kind of interaction can also lead to surface erosion. The latter process is often referred to as electronic sputtering [12]. This is a common process in space where, for example, icy objects are eroded as a result of energetic ion impact.

3.2. Electronic sputtering and ion tracks

When an ion with a velocity higher than the Bohr velocity interacts with a solid, ion–electron collisions dominate. Close to the trajectory of the incoming ion, a very high electronic excitation density is created by fast heavy ions producing the so-called infra track. Further away from the track's axis, the energetic secondary electrons, which had been released in direct ion–electron collisions, slow down and create a region with lower energy density called the ultra track. The size of this region is defined by the projected range of the energetic secondary electrons. The conversion of electronic excitation energy into mechanical and chemical energy links atomic physics in solid at low excitation densities to nanometer-scale continuum mechanics at high excitation densities. The energy gradient sets up a pressure pulse in the solid. At the cross-section of the particle track with vacuum this pressure pulse is believed to produce a coherent ejection of relatively large pieces (many weakly interacting atoms like in a cluster or a molecule) of material [12].

3.3. Fast ion induced desorption of biomolecular ions

Fission fragments from a Californium source are examples of fast heavy ions interacting with a solid in the electronic stopping regime. Ron Macfarlane and co-workers [6] found already in 1974 that fission fragments bombarding a surface covered with a layer of biomolecules like small peptides could cause ejection not only of fragment ions from these molecules but also, more surprisingly, ejection of whole intact molecular ions. Macfarlane called the method plasma desorption mass spectrometry (PDMS), thereby associating the plasma created by the fast ion in the infra track of the fission fragment in a solid to a desorption process. PDMS subsequently developed into a technique that was applied to the study of many biomolecules, see Section 2.

3.4. The erosion process

At the time Macfarlane made his discovery, the erosion process was not understood as an electronic sputtering process. In fact, for many years the erosion process was not studied at all. Salehpour et al. [13] performed the first systematic studies of the erosion of a multilayer of the amino acid leucine induced by fast heavy ions. The sputtered, neutral biomolecules were collected, and the material on the collector was subsequently analyzed for its amino acid content. The first experiment showed that the total erosion yield of neutrals from the amino acid leucine bombarded with 90 MeV ^{127}I was of the order of 1000 molecules per impacting ion. The typical ratio of desorbed ions to neutrals deduced from that study was 1:10,000. Later Hedin et al. [14] showed that the total erosion yield scaled with the electronic energy density (electronic stopping power) in the ion track to the third power, see Fig. 6. The velocity of the various primary

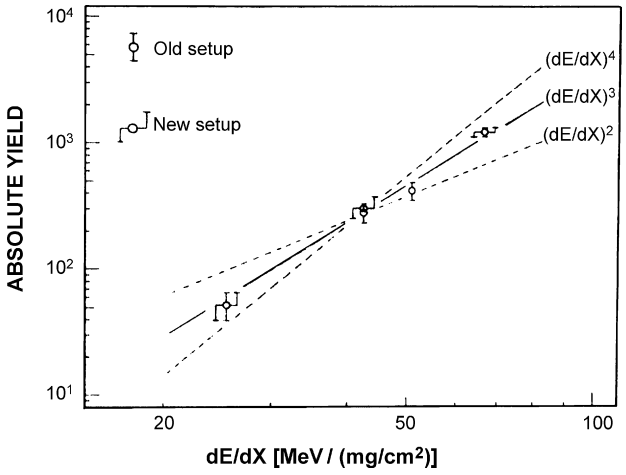

Fig. 6. The sputtering yield of neutral, intact, leucine molecules as a function of the electronic stopping power. The three lines shown are the scaling with the indicated powers of the stopping power of the primary ions. [Reprinted figure with permission from A. Hedin, P. Håkansson, M. Salehpour, B.U.R. Sunqvist, Phys. Rev. B 35 (1987) 7377. Copyright 1987 by the American Physical Society.]

ions used was kept constant so that energy density scaled as the electronic stopping power of the primary ions.

In a later study, Kopniczky et al. [15] used the emerging technique atomic force microscopy (AFM) to study the craters formed in L-valine crystals as a result on the impact of 90 MeV ^{127}I ions, see Fig. 7. The AFM studies confirmed the neutral yields and their dependence of electronic stopping power of the bombarding particle as suggested in Ref. [14].

3.5. Angular distributions of ejected molecular ions

In a study of ejected secondary ions from various large peptides and small proteins, Ens et al. [16] found that intact

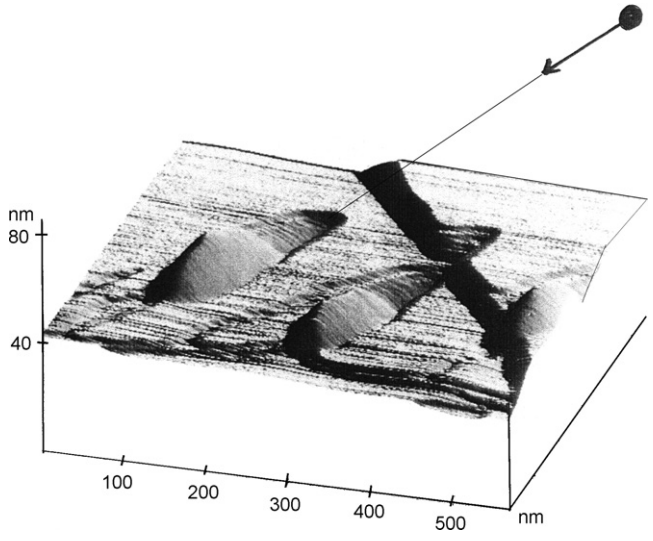

Fig. 7. Erosion formed by 90 MeV ^{127}I ions bombarding an L-valine crystal surface at grazing angle of incidence. It is clearly seen how the ion starts to plow down into the surface. Some material at the end forms a hillock. When the angle of incidence becomes perpendicular to the surface a crater is formed at the point of impact. [Reprinted with permission from Judit Kopniczky, Licentiate Thesis, Department of Physical Chemistry, Uppsala University, 2001.]

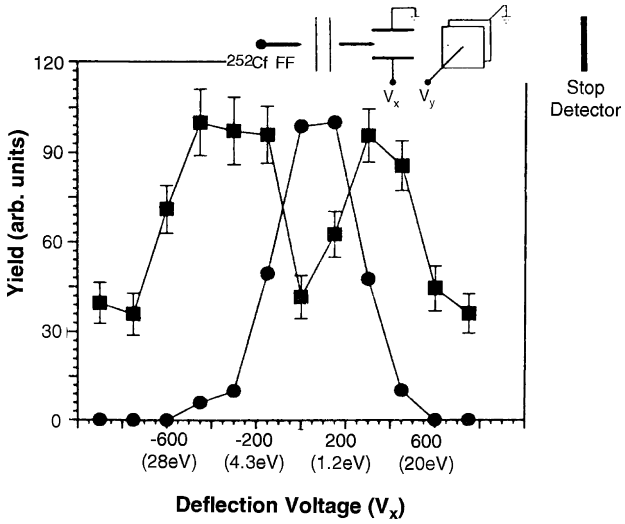

Fig. 8. Relative yields of positive molecular ions of bovine insulin (squares) and CH_3 ions (circles) emitted when a sample of bovine insulin was bombarded with fission fragments from behind at normal incidence as a function of defelction voltage in a direction perpendicular to the direction of the secondary ions. The numbers in parenthesis on the x-axis give $(1/2)\,m v_r^2$ where v_r is the radial velocity of bovine insulin ions which strike the centre of the detector with the corresponding deflection voltage. [Reprinted with permission from W. Ens, B.U.R. Sundqvist, A. Hedin, P. Håkansson, G. Jonsson, Phys. Rev. B 39 (1989) 763. Copyright 1989 by the American Physical Society.]

molecular ions of insulin are ejected from the surface at an angle which correlates with the incident ion direction, see Fig. 8. The experiment indicated that from the ionized central part of the cylindrical region, the infra track, there is a direct radial momentum transfer, i.e., a non-diffusive process, which ejects large molecular ions. The ions are pushed out from the surface by the expanding track. The asymmetry of the ejected plums was explained as caused by the radial component of the expanding track core.

3.6. The pressure pulse model

The energy density in the track corresponds to a radial gradient, and any energy density gradient will set up a radial pressure [12]. Such a pulse will give rise to an erosion process. This was simulated in a molecular dynamics calculation [17], which also reproduced the scaling with electronic stopping power as found in Ref. [14]. The results of the simulation inspired the development of an analytical model, the pressure pulse model [18], with which the aforementioned experimental scaling of neutral leucine-yields with the electronic stopping power of the incident ion and mean ejection angles of ejected molecular ions could be reproduced. This model provided a plausible description of the basic mechanism involved in fast heavy ion induced desorption (PDMS), see Fig. 9. The high energy and ionization density in the inner part of the track leads to fragmentation and ejection of hot, small fragments and fragment ions. The angular ejection pattern of those ions are discussed below. The larger molecular ions were ejected further out in the track, where the pressure pulse acts coherently on many atoms in the solid and, therefore, accounts for the special feature of the

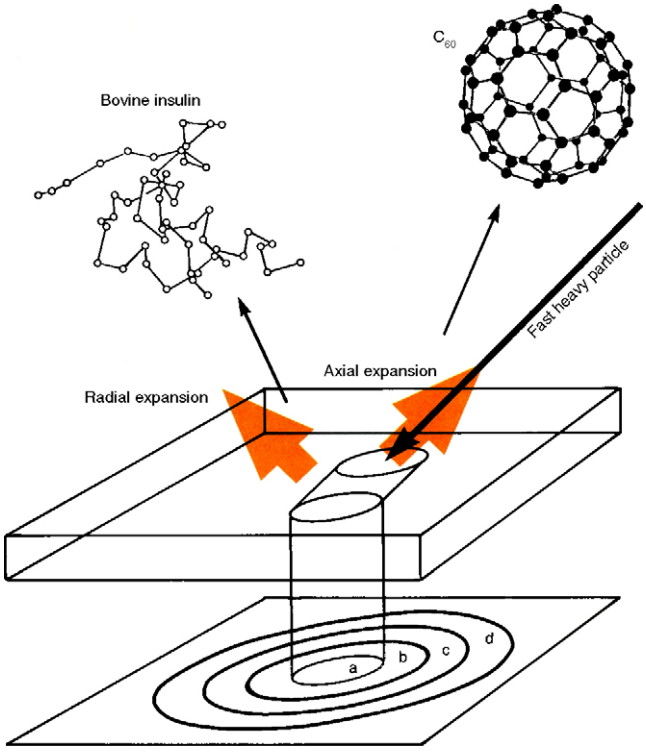

Fig. 9. Fast heavy ions can eject an intact bovine insulin molecule ($C_{254}H_{377}N_{65}O_{75}S_6$) from a solid sample of bovine insulin as well as eject a "bucky ball" (C_{60}) from $(C_2H_2F_2)_n$. Bucky balls and other molecules are formed in the highly ionized region a, which has a radius of about 0.4 nm for a 1 MeV/u incident ion. Large intact ions are ejected from region b (outer radius, 1–2 nm); large intact neutrals from region c (about 4 nm). Damage from the impact extends out to region d (about 10 nm). [Reprinted figure with permission from R.E. Johnson, B.U.R. Sundqvist, Physics Today, March (1992) 28. Copyright 1992 by the American Institute of Physics.]

process, namely the ejection of whole molecular ions from the surface.

3.7. Infra track sputtering

The beauty of ion track physics is that the effects caused by a single ion impacting a surface can be studied. The fast heavy ion can be used to mark the start of an erosion process. The ejected ions can then be studied with time-of-flight mass spectrometry, and the resulting crater can be visualized with a method like AFM.

Papaleo et al. [19] studied the small hydrocarbon fragments ejected when a biomolecular solid is bombarded with fast heavy ions. Using the high mass resolution of a mirror time-of-flight instrument, it was found that the small fragment ions ejected from the surface are formed closer to the track than the larger and more complex secondary ions. The bare carbon-cluster ions compared to those fragments with larger and larger numbers of attached hydrogen atoms are formed closest to the fast ion track. The carbon ions are even ejected at an angle, as if pushed out by the axially expanding track core.

Papaleo et al. [20] were also able to show that radial velocities and effective ejection radii of hydrocarbon secondary ions sputtered from polymer films could be experimentally determined. Radial profiles of the energy and momentum density in an individual MeV ion track could be directly mapped out on a picosecond time scale.

Another exciting feature of the infra track is that secondary ions of C-60 was formed in tracks from polyvinylidene difluoride (PVDF) films [21]. The fullerene ions were preferentially ejected in the direction back along the direction of the primary ion. This indicates that the fullerenes are produced in the hot plasma formed in the infra track of the primary particle. It is remarkable that the C–C bonds are so energetically favoured that a rapid local collapse of the material occurs during the net outward expansion. The volume occupied by the 60 carbons in the original material is about eight times larger than that of vibrationly relaxed C_{60}.

In summary, the original discovery by Macfarlane and co-workers of what they called plasma desorption mass spectrometry inspired the Uppsala Ion Physics group to study the electronic sputtering from organic solids and to develop a physical understanding of the ejection process.

4. Modern protein mass spectrometry in Uppsala

4.1. The first initiatives of the Odense–Mölndal–Uppsala axis

During the same period in which very strong, fruitful research in mass spectrometric techniques was taking place in Uppsala, a number of researchers active in clinical neuroscience and particularly in neurochemistry at Sahlgrenska University Hospital, Mölndal, realized a great need for more accurate methods for the analysis and characterization of molecular species in complex mixtures. Rolf Ekman, his graduate student Jonas Bergquist, and other co-workers in Mölndal and Göteborg initiated a collaboration with both Peter Roepstorff in Odense and Per Håkansson in Uppsala. At this time, in the late 1990s the method of choice for detection and quantitation of neurotransmitters (such as neuropeptides) was the radioimmunoassay (RIA) with its inherent limitations and possible flaws due to cross-reaction. The RIA methods were more and more replaced by enzyme linked immunosorbent assays (ELISAs) in order to circumvent the necessity of using radioactively labeled compounds. However, ELISA still struggles with possible cross-reactivity and nonspecific binding to surfaces. Mass spectrometry offered a major breakthrough in this research as the techniques became more and more accessible to non-expert users. Mass spectrometry was, however, not a totally new technique at Sahlgrenska and Göteborgs University thanks to the very early initiatives by the Stenhagen couple. At that time, however, mass spectrometry mainly focused on smaller endogenous compounds such as various fatty acids and glycosylations. Protein and peptide mass spectrometry was rather scarce. Both Peter and Per gave important support and advice for an investment in the first MALDI-TOF MS instrument (a Bruker Reflex II) at Mölndal Hospital. This MALDI-TOF MS instrument made it possible to address many intriguing questions concerning the molecular character of various biological tissues and fluids. Some applications called for more accurate mass determina-

tion, e.g., in the identification of an unexpected protein family, the defensins, when they were first discovered in human lymphocyte nuclei [22]. Per Håkansson and co-workers contributed to this latter study by performing numerous analyses on their 9.4 T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker 9.4T BioAPEX-94e FTICR MS), which at the time had been recently installed, as well as by sharing their vast knowledge in ion physics and high mass resolution mass spectrometry. This collaboration was initiated in parallel with one between Mölndal and Odense that produced in an exchange graduate student Johan Gobom, who ended up spending most of his graduate time in Denmark. One of the fruitful projects to emerge from the Mölndal Odense collaboration was an effort to isolate and characterize nuclear associated proteins in human lymphocytes [23]. The combination of a relevant biological and clinical question, a thorough sample preparation, a method for high-resolution 2D-gel electrophoresis, and a frontline mass spectrometric capability made this study possible.

4.2. Analytical chemistry and ion physics join forces in protein mass spectrometry

During 1999, Jonas Bergquist was recruited to Uppsala University to help bridge the gap between medical and natural sciences. He joined the group of Karin Markides in Analytical Chemistry, a very strong and well-recognized environment for miniaturized liquid-based separation and mass spectrometric detection. From the very beginning, an intriguing research project was established together with Per Håkansson and his graduate student Magnus Palmblad using the FTICR instrument; this study resulted in a number of breakthrough papers in protein mass spectrometry. The first was a demonstration that the high resolution and mass accuracy inherent in FTICR MS made possible the analysis of tryptic digests of protein mixtures without any separation [24]. First, the method was demonstrated on a mixture of tryptic digests of equine cytochrome *c*, equine myoglobin, and bovine serum albumin. The same method was then applied to human plasma from a healthy blood donor. Computer programs were employed to simplify analysis of the complex spectra. The 2745 peaks in the human plasma electrospray ionization FTICR spectrum could be reduced to 1165 isotopic clusters and 669 unique masses, see Fig. 10. Out of these, the masses of 82 were assigned with mass measurement errors less than 10 ppm; these assignments matched tryptic fragments of serum albumin that covered 93% of the sequence. Another 16 masses were assigned to tryptic fragments of transferrin, covering 41% of the sequence at the 10 ppm mass measurement error level (14 within 2 ppm). The mass measurement errors were approximately normally distributed with a standard deviation of 1.7 ppm. This demonstrated the feasibility of combining the ultra-high mass resolving power and accuracy of FTICR mass spectrometry with automated computer analysis for investigating complex biological matrices. The great advantages of using high-mass resolution in combination with the high-mass accuracy that FTICR MS offers in clinical applications, like fast screening of human body fluids [25], were also identified at that time.

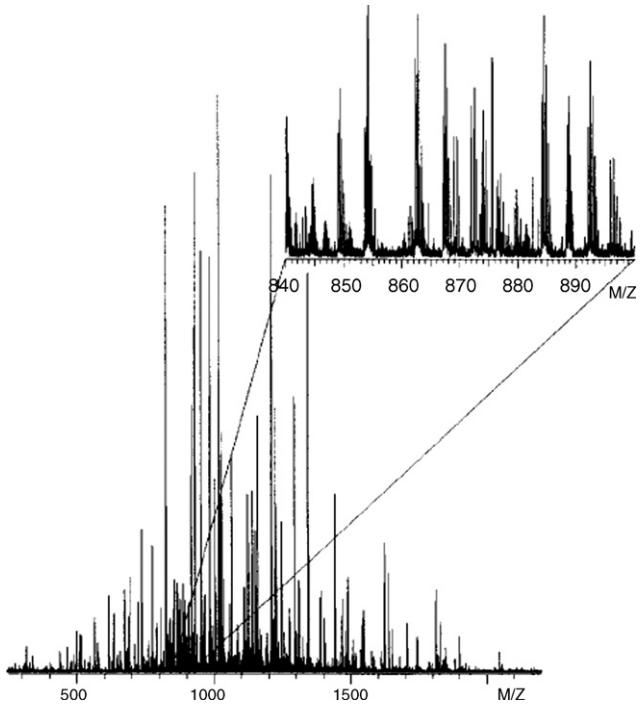

Fig. 10. Mass spectrum of human plasma. Two thousand seven hundred and forty-five peaks found could be reduced by computer analysis to 1165 isotopic clusters and 669 unique masses. The expansion shows the region *m/z* 840–900. [Reprinted with permission from Ref. [24]. Copyright 2000 John Wiley & Sons Ltd.]

4.3. On-line liquid separation electron capture dissociation FTICR MS

The next ground-breaking findings were presented as a result of the first successful on-line coupling of liquid chromatography and electron capture dissociation (ECD) [26] in Fourier transform ion cyclotron resonance mass spectrometry in collaboration with a student in the Ion Physics group, Youri Tsybin [27]. This hyphenated technique was used for the analysis of peptides and digested proteins and also used a novel injection system to increase the yield of fragmentation. This tool although in an early stage showed qualities that were attractive for proteomics studies and especially in the mapping of posttranslational modifications. Also combined with an even faster separation technique – capillary electrophoresis (CE) – the ECD technique has been proven to function as an efficient fragmentation technique [28]. A number of studies were performed by Magnus Wetterhall in order to further take the full advantage of the fast separation that CE offered for extremely complex samples and we managed to show that even when a minute sample amount was injected a fast and accurate survey of the protein content of human cerebrospinal fluid (CSF) could be obtained with the FTICR [29], see Fig. 11.

4.4. Clinical proteomics and pattern fingerprints using LC-FTICR MS

A new graduate student, Margareta Ramström, was recruited as a shared student between Analytical Chemistry and Ion Physics. Margareta explored the advantages of performing

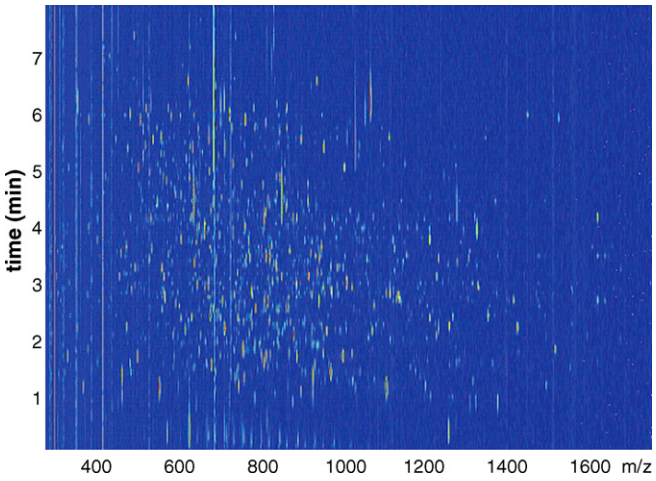

Fig. 11. Two-dimensional view of a CE–FTICR MS analysis of a CSF tryptic digest. A total of 10,140 peaks could be reduced to 1497 unique masses. The redundancy is due to presence in consecutive spectra, multiple charge states, and multiple isotopic peaks. [Reprinted with permission from Ref. [29]. Copyright 2002 American Chemical Society.]

on-line capillary liquid chromatography and FTICR MS for the analysis of complex clinical samples [30]. Margareta also showed for the first time the great potential of combining ECD and nozzle scimmer fragmentation information from on-line LC–FTICR MS in the analysis of biological samples [31]. In the study of isolated pancreatic islets, Margareta's approach made it possible to identify two new secreted hormonal peptides. The extent of the complementary information one can obtain from LC-ion trap and LC–FTICR MS when studying complex samples was also shown in collaboration with Bill Hancock's group [32]. The continual, simultaneous development of techniques for sample preparation, separation, and detection together with the very important design of a multivariate data analysis tool for evaluation of generated mass-chromatograms or individual pattern fingerprints, see Fig. 12, made it possible in a collaborative effort with a clinic to present the first thorough comparison of CSF from patients with amyotrophic lateral sclerosis and matched healthy controls [33]. With this approach it was possible to identify and classify some biomarkers present in cerebrospinal fluid which represent an on-going neurodegenerative process. These findings were important not only as a possible target for early diagnostics but also because they could shed some light on the pathophysiological basis of the disease and, potentially, generate ideas about new targets for interventions.

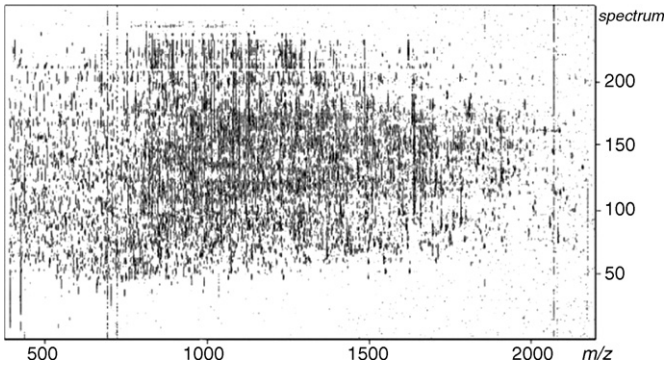

Fig. 12. Mass chromatogram of a tryptic digest of CSF. The mass-to-charge ratio is shown on the *x*-axis and the time of elution on the *y*-axis. On average around 3700 peptides were detected in one experiment. [Reprinted with permission from Ref. [33]. Copyright 2004 Wiley-VCH Verlag GmbH & Co. KGaA.]

4.5. Quantitative proteomics, selective depletion and focused tissue proteomics

Charlotte Hagman was the second shared student between the Analytical Chemistry and Ion Physics groups; she focused on the very important issue: quantitative measurements in complex mixtures using the global labeling approach [34]. Charlotte also worked in collaboration with Margareta to reduce the levels of the most abundant proteins in body fluids by selective depletion [35]. This step is a really needed in order to be able to look deeper into the proteome of complex samples. Yet another approach is to aim for a very exclusive selection of a sub-proteome by isolating single cells or minute amounts of tissue from clinically relevant areas of the body. In a rather recently published study, we show that by the use of laser microdissection methodology it is possible to study differences in protein expression in minimal amounts of sample [36]. As a very important step in this approach the third shared student, Jörg Hanrieder, has been recruited. Yet another possibility for more focused proteomics is to use the small pieces of tissue normally discarded when patients undergo heart surgery and are placed in extra corporal circulation [37].

4.6. Current status of the clinical proteomics initiative in Analytical Chemistry, Uppsala

From a modest start, thanks to Peter Roepstorff, we have developed a rather fruitful line of research in modern biologically and clinically driven mass spectrometry. Jonas Bergquist, now professor in Analytical Chemistry and neurochemistry, heads a group of >30 including senior researchers, lecturers, graduate and undergraduate students. The current developmental aim is to refine the coupling of sampling, sample preparation, liquid-based multidimensional separation including isoelectric focusing, capillary liquid chromatography, capillary electrophoretic and capillary electrochromatographic separation to high resolution mass spectrometry including FTICR MS, ESI-TOF MS, ESI-Qtrap MS and MALDI-TOF/TOF MS for the analysis of clinical samples in search for relevant biomarkers.

5. A link between tandem mass spectrometry of peptides and protein structure in solution

One of the beautiful features of mass spectrometry is that it seamlessly combines fundamental and applied aspects. As an example, high-throughput proteomics work that otherwise could have been compared to a "stamp collection" provides ample opportunity for studying the mechanisms of ion fragmentation. A library of some 15,000 peptide MS/MS spectra acquired with collision activation during proteomics work allowed us to test the

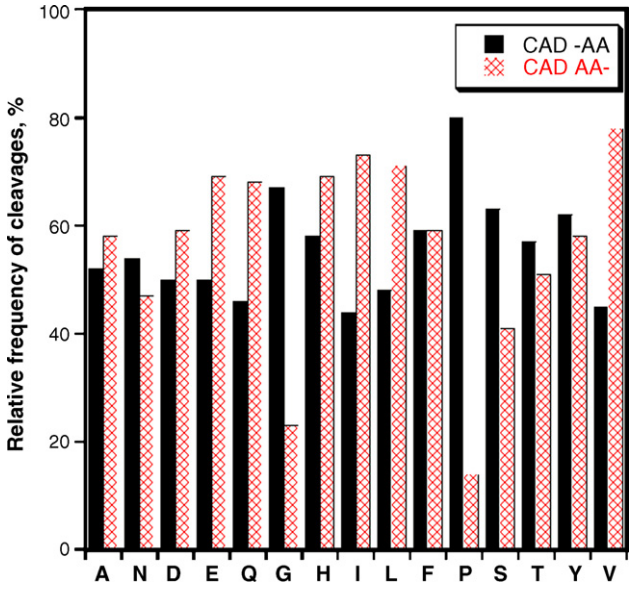

Fig. 13. Relative propensities for cleavage before (-AA) and after (AA-) an amino acid (AA) in collision activation dissociation for backbone C—N cleavages removing three to five amino acids from the N-terminus.

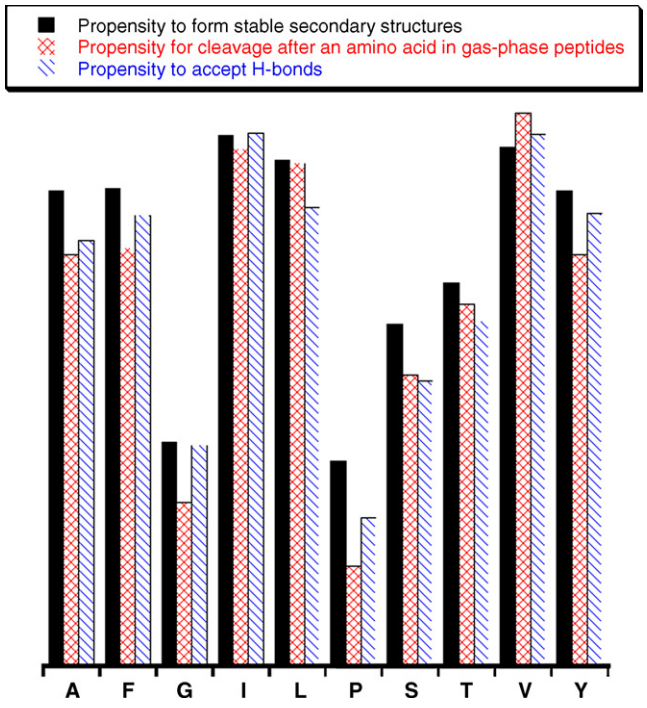

Fig. 14. Relative propensity of an amino acid carbonyl to accept hydrogen bonds, to participate in stable secondary motifs (α-helices and β-sheets) and gas-phase propensities for cleavage after an amino acid in collision-activated dissociation. The first two propensities are calculated from an X-ray structure database containing thousands of proteins, the latter propensity is experimentally measured by mass spectrometry.

activation mechanism, see Fig. 13. It can be hypothesized that the most important factor determining the cleavage site probability after an amino acid residue A must be the carbonyl basicity of that residue [38]. In order to test this hypothesis, one needs to evaluate whether positive correlation exists between the cleavage frequency after A with the $GB_{carbonyl}(A)$. But the values of $GB_{carbonyl}$ have been absent in literature.

To address this issue and derive the $GB_{carbonyl}$ values, a database of X-ray protein crystal structures was interrogated. When a polypeptide chain folds into secondary structure, the main forces holding it together are due to hydrogen bonding, in which backbone carbonyls act as acceptors. The ability of a given carbonyl to form hydrogen bonding and thus to stabilize the secondary structure directly relates to its basicity. Thus by calculating the frequency of hydrogen bond acceptance for a given type of amino acid one can estimate its carbonyl basicity.

There is another, independent way of estimating $GB_{carbonyl}(A)$, as the frequency of participation of a given amino acid in most stable secondary structures, α-helices and β-sheets, must also relate to carbonyl basicities. In agreement with this suggestion, an excellent correlation was found between the estimates of $GB_{carbonyl}(A)$ made through the frequencies of H-bond acceptance and through the secondary-structure participation frequencies, see Fig. 14 [39]. Each of these estimates correlated well with the probability of peptide bond cleavage in collisional activation.

Thus, the hypothesis of the prime role of carbonyl basicity in the gas-phase bond cleavage in peptides was confirmed using X-ray data acquired on solid-phase protein crystals. Moreover, spectrometric analyses uncovered a direct link between the frequency of H-bond acceptance and the participation frequency in most stable motifs of the secondary structure. And finally, mass spectrometry provided a tool for predicting the effect of new amino acids or backbone linkers on protein structure without producing a full-size protein, crystallizing it, and recording its X-ray image.

6. Conclusions

It is obvious that modern mass spectrometry, protein mass spectrometry in particular, has moved from being an area of interest for highly specialized researchers in ion physics to an important tool in chemistry, biology, pharmacy and medicine. This development was made possible by the continuous improvements of methods and instrumentation and the commercialization of these new tools. Another important contributing factor to this development is the collective cross-disciplinary effort which has characterized the field. Peter Roepstorff epitomizes this spirit with his ever present enthusiasm for collaborations, especially those that cross-disciplinary boundaries.

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