Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 620–623, February 1987
Chemistry

Tandem quadrupole Fourier-transform mass spectrometry of oligopeptides and small proteins

(liquid secondary-ion mass spectrometry/melittin/glucagon/insulin/cytochrome *c*)

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Communicated by Fred W. McLafferty, September 29, 1986 (received for review August 10, 1986)

ABSTRACT **Modifications to the newly developed tandem quadrupole Fourier-transform mass spectrometer have made it possible to record mass spectra on oligopeptides and small proteins in the mass range between 2 and 13 kDa.**

Methodology for the sequence analysis of proteins by tandem mass spectrometry has been under development in our laboratory for several years (1, 2). Presently this approach involves enzymatic and/or chemical degradation of the protein to a collection of peptides that are then fractionated by high-performance liquid chromatography. Each of 20 to 40 fractions containing as many as 10 to 15 peptides is then analyzed directly without further purification by a combination of liquid secondary-ion/collision-activated dissociation mass spectrometry on a triple-quadrupole instrument. Ions characteristic of the molecular mass of each peptide in a particular fraction are selected one after the other by the first quadrupole analyzer, these ions are dissociated by collision with argon atoms in the second quadrupole, and the masses of the resulting fragments are analyzed in the third quadrupole. The result is a collection of mass spectra characteristic of the amino acid sequence in each of the peptides produced in the enzymatic digest of the original protein. From an initial 10 nmol of 50-kDa protein, it is usually possible to obtain sequence information covering 25% to 60% of the sample in the 4 to 5 days required to do the above biochemical and instrumental manipulations. The major limitation of this approach has been the 1.8-kDa mass upper limit of our triple-quadrupole instrument. Maximum sequence information is obtained only when the protein under investigation is cleaved efficiently into peptides of molecular mass under this ceiling.

To extend the methodology to mixtures of larger oligopeptides, we have recently constructed a tandem quadrupole Fourier-transform mass spectrometer (3, 4), similar to that described by McIver *et al.* (5). This instrument is equipped with a 7-T superconducting magnet and operates with a mass range in excess of 20 kDa. Fourier-transform instruments have tremendous potential for the analysis of large biological molecules (6–8), because (*i*) they record the masses of all ions in the spectrum simultaneously and do not have to be scanned while the sample is continuously consumed during ionization; (*ii*) they function as ion storage devices that permit accumulation of ions produced in low abundance from small amounts of sample; (*iii*) they facilitate direct analysis of oligopeptide mixtures by the double-resonance technique (8); and (*iv*) they appear to be ideally suited for collision-activated dissociation (8) or laser photodissociation experiments (9) that produce fragment ions carrying sequence information from oligopeptide $(M+H)^+$ ions.

One disadvantage of Fourier-transform mass spectrometers is that they must be operated at a pressure less than 10^{-8} torr (1 torr = 133 Pa) in the analyzer to prevent collisions between ions and neutral gas molecules from interfering with the mass measurement process. This requirement has made it difficult to use Fourier-transform instruments in conjunction with volatile liquid matrices employed in the highly successful particle-bombardment ionization techniques for biological molecules. Problems associated with high gas flow during sample ionization are circumvented in the tandem quadrupole Fourier-transform instrument, because sample introduction and ionization take place in a differentially pumped quadrupole ion source, and only the ions of interest are transferred through the fringing fields of the superconducting magnet and into the ion-cyclotron resonance (ICR) cell for mass analysis (3–5).

Another problem associated with Fourier-transform instruments stems from the time scale required for mass analysis. Spectra showing unit resolution over the mass range from 0.1- to 10-kDa can only be obtained if the cyclotron motions of ions trapped in the cell can be monitored for several hundred milliseconds. This, in turn, requires that a substantial fraction of the sputtered $(M+H)^+$ ions be stable toward fragmentation over this time frame. This situation is to be contrasted with that for time-of-flight and magnetic-sector instruments where the time between ion formation and ion detection is less by a factor of thousands than that on a Fourier-transform mass spectrometer.

During the course of the present study, insulin $(M+H)^+$ ions desorbed from the solid state using 100-Mev fission fragments as projectiles were reported to undergo extensive dissociation on both the nanosecond and microsecond time scale (10). If the same fragmentation phenomena were to be observed on bombardment of a liquid matrix with keV projectiles, molecular mass determinations on large biological molecules by Fourier-transform mass spectrometry might not prove possible. Here we show that such determinations are possible; mass spectra are reported on a number of oligopeptides and small proteins ranging in mass up to 13 kDa.

MATERIALS AND METHODS

Tandem Quadrupole Fourier-Transform Mass Spectrometer. Earlier versions of this instrument have been described (3–5). The instrument in its present configuration is shown schematically in Fig. 1. The ion source, lens system, first quadrupole mass filter, and the electronic equipment to

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Abbreviation: ICR, ion cyclotron resonance.
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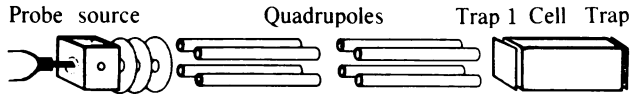

FIG. 1. Diagram of the tandem quadrupole Fourier-transform mass spectrometer.

operate all of the above were purchased from Finnigan-MAT (San Jose, CA); these are standard items on their model 4500 quadrupole mass spectrometer. The length of the first quadrupole mass filter has been increased from 15 cm to 25 cm. To reduce gas pressure in the vicinity of the sample probe, most metal in the ion-source block has been removed; only that part required to mount the ion source onto the lens structure has been retained. The model 4500 spectrometer electronics that drives both quadrupole 1 and 2 have been modified to operate at 870 kHz. In the rf-only mode, the system functions with a high-mass cutoff well above mass 10 kDa and a low-mass cutoff that can be set anywhere below mass 3 kDa.

The remainder of the instrument consists of a second set of quadrupole rods that are 86.25 cm long, an ICR cell constructed from highly polished stainless steel plates, 7.75 × 2.8 × 2.8 cm, and a 7-T superconducting magnet with a 15-cm-diameter bore from Oxford Instruments (Oxford). Trapping plates 1 and 2 in Fig. 1 have the dimensions 2.8 × 2.8 cm and contain 1.75-cm^2-square holes that function as entrance and exit apertures to the cell; these apertures are covered with 90%-transmission nickel screen. During operation under conventional conditions for liquid secondary-ion mass spectrometry, the pressure in the ICR cell is maintained at (0.5–3.0) × 10^{-9} torr by three cryogenic pumps (model HV-202-6C) cooled by two compressors (model 204SL) from Air Products (Allentown, PA). The estimated pumping speed for each of these units is 680 liters of air per min. The three pumps are placed below the ion source, below quadrupole 1, and below a point just beyond the entrance to the second set of quadrupoles that lead into the ICR cell, respectively. Pneumatically operated high-vacuum gate valves (model 10044-UE40) from VAT (Woburn, MA), separate each cryogenic pump from the vacuum manifold. A model 2000 electronics/data-system package from Nicolet Analytical Instruments (Madison, WI) is used to acquire and process data from the ICR cell.

Sample ionization is accomplished by using a liquid matrix and a cesium ion gun (Antek, Palo Alto, CA) (11). In this gun the cesium ion source serves as the anode and floats at variable potential, typically 6–10 keV. The anode is separated from the liquid matrix by extractor and focusing lenses that float at 95% and 90% of the anode potential, respectively. To minimize sample consumption, the above system has been modified to operate in the pulsed-mode under control of the Nicolet model 2000 data system. Cesium ions are generated continuously but are pulsed onto the sample probe by switching the potential of the extractor lens from a value 105% to 95% that of the anode. The width of the pulse can be set as low as 1 msec, while the pulse frequency operates as high as 200 Hz.

Biological Samples. Neurotensin, melittin, glucagon, bovine insulin, porcine insulin, and horse cytochrome *c* were obtained from Sigma and were purified by reverse-phase high-performance liquid chromatography on a C_{18} column from Waters (Watertown, MA). Gradient elution with either 0.1% trifluoroacetic acid/0.05% trifluoroacetic acid-acetonitrile or 5% (vol/vol) acetic acid/1-propanol mixtures was used for this purpose.

Operation of the Tandem Quadrupole Fourier-Transform Mass Spectrometer. All samples used in the present study were prepared by adding 0.5 μl of a 200 pmol/μl solution of peptide in 5% acetic acid to a mixture containing 0.5 μl of thioglycerol/glycerol 1:1 and 0.5 μl of 6 M HCl in dimethyl sulfoxide on a gold-plated, stainless-steel probe. A positive potential of 1.5 V was applied to the sample probe after it had been inserted into the Finnigan model 4500 ion source. Bombardment of the sample matrix with a 4-msec pulse of 10-keV Cs^+ projectiles was employed to sputter sample and matrix ions into the gas phase. Quadrupoles 1 and 2 were operated in the rf-only mode with an offset potential of −20 V and a low-mass cutoff at 0.4 kDa. Under these conditions, the quadrupoles pass all ions above m/z 400 and reject most of the abundant low-mass ions derived from both the sample and liquid matrix. The latter would quickly exceed the capacity of the ICR cell and impair the ability of the instrument to detect weaker signals at the high-mass end of the spectrum. The potential applied to trapping plates 1 and 2 on the cell was +3 V; receiver and transmitter plates were maintained at 0 V potential.

After the above parameters were set, mass spectra were acquired by using a series of pulses controlled by the data system. The first, or quench pulse, applied potentials of +10 and −10 V to the last trapping plate and was used to clear the cell of all charged particles. The second, or ionization pulse, lowered the voltage on the extractor lens of the Cs^+ gun and allowed 10-keV Cs^+ ions to impact on the sample matrix for 4 msec. The same pulse placed rf on the two sets of quadrupole rods and lowered the potential on trapping plate 1 to 0 V for the identical 4-msec period. A sweep-out pulse to remove unwanted ions at low mass from the cell was not used in the present study. After a 10-msec delay, a rf pulse of 35 V peak-to-peak and 2.5-msec duration was applied to the ICR cell containing the trapped ions. This pulse contains a range of frequencies that accelerates all ions of all masses and causes them to move coherently at their characteristic cyclotron frequencies. Ion image currents induced by the ions on the cell receiver plates were monitored for 8 or 16 msec, amplified, and digitized. Data from 20 to 200 such experiments were acquired, summed together, and the resulting time-averaged signal was then converted to the desired mass spectrum by using a 16- or 32-K Fourier-transform. Total experiment time to produce the required data was 0.6–6 sec. Total time for sample ionization was 0.08–0.8 sec.

RESULTS AND DISCUSSION

Mass analysis by Fourier-transform mass spectrometry of oligopeptide ions desorbed from a liquid matrix under Cs^+ ion bombardment has been described (3, 4). Ions characteristic of molecular weight for samples in the range between 2000 and 3500 had been observed but with poor resolution and low signal/noise ratio. Here we report greatly improved performance as a result of two modifications of the tandem quadrupole Fourier-transform instrument. The first modification involved "milling out" the ion source block to increase the pumping speed and reduce the gas flow in the vicinity of the sample probe. The importance of gas pressure on the performance of particle-bombardment ion sources has been discussed by Aberth (12). Pulsing the voltage on trapping plate 1 to 0 V during the ionization event constituted the second modification. As a result of these changes, the amount of sample consumed to produce excellent quality spectra on oligopeptides below mass 2000 has been reduced by a factor of more than 250. More importantly the useful mass range of the instrument for characterizing the molecular weights of oligopeptides and small proteins has been extended beyond mass 10,000.

Shown in Fig. 2*A* is the mass spectrum of the tridecapeptide neurotensin obtained by bombarding a 100-pmol sample in a thioglycerol/glycerol matrix with 10-keV Cs^+ ions for 4 msec. Sample ions sputtered from the matrix under these conditions are transferred through the fringing magnetic

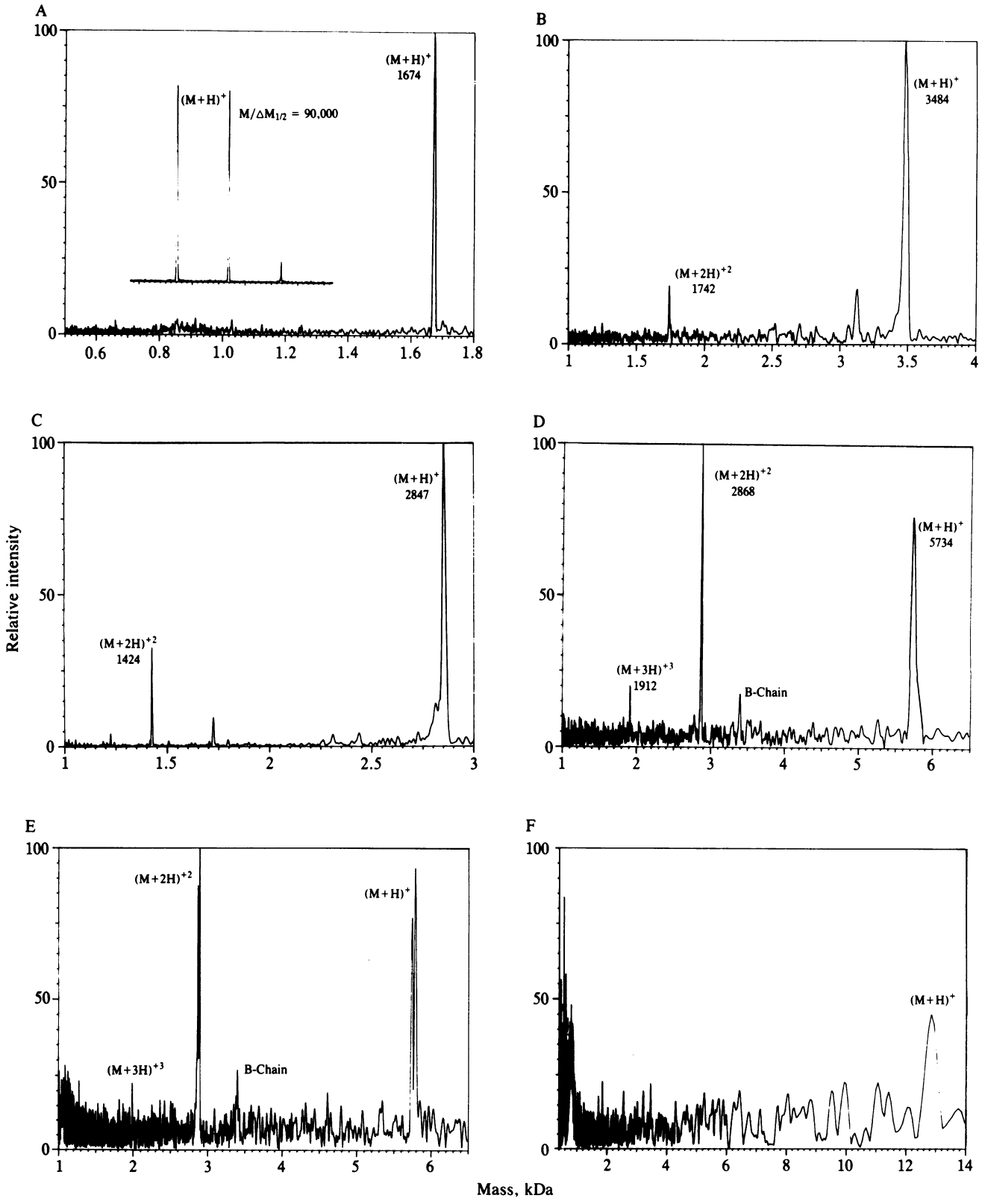

FIG. 2. Liquid secondary-ion mass spectra. (*A*) Neurotensin (100 pmol); M_r = 1672.9. (*B*) Glucagon (100 pmol); M_r = 3482.8. (*C*) Melittin (100 pmol); M_r = 2846.5. (*D*) Bovine insulin (100 pmol); M_r = 5733.5. (*E*) Bovine (M_r = 5733.5) and porcine (M_r = 5777.6) insulins (200 pmols each). (*F*) Horse cytochrome *c* (M_r = 12,384).

fields of a 7-T superconducting magnet with the aid of two sets of rf-only quadrupole rods and become trapped in an elongated ICR cell. In the present study the rf power on the quadrupole rods was set so as to reject all ions below mass 400. Voltage on trapping plate 1 was set at 0 V during ion transmission and raised to +3 V at the end of the ionization period to enhance the trapping efficiency of the cell. As soon as the ions enter the cell, they are excited by an rf pulse containing a range of frequencies that causes them to move coherently in large orbits at their characteristic ion-cyclotron frequencies. This motion produces image currents on the two receiver plates that are then amplified, digitized, and Fourier-transformed. Data from 20 such experiments were summed to generate the spectrum in Fig. 2*A*. Although strong ion current

can usually be obtained from a 100-pmol sample for several tens of seconds, the total ionization time required to obtain the signal/noise ratio shown in Fig. 2*A* was only 80 msec. Thus only a small fraction of the 100-pmol sample was consumed in the above experiment. The spectrum shown in the insert to Fig. 2*A* was taken on the same 100-pmol sample and is indicative of the excellent resolution that can be achieved at this mass by using an excitation pulse of 1-kHz bandwidth and by monitoring the image current for ions in the corresponding 0.02-kDa mass range for 2–4 sec.

Shown in Fig. 2 *B* and *C* are mass spectra recorded on two peptides in the mass range between 2- and 4-kDa. Melittin and glucagon, 26- and 29-residue peptides, respectively, were examined in an earlier paper (4). Both molecules afforded $(M+H)^+$ ions with a signal/noise ratio <10, and then, only when subjected to Cs^+ ion bombardment for 5 sec. In contrast, spectra obtained on the newly modified instrument required a total ionization time of only 80 msec and yet exhibit excellent signal/noise ratios.

Fig. 2*D* shows the mass spectrum of bovine insulin obtained by summing the results of 200 experiments in which the same liquid matrix-containing 100-pmol sample was bombarded with 10-keV Cs^+ ions for 4 msec. The total ionization time used for this experiment was 800 msec. Experimental time for sample introduction, ionization, data acquisition, data reduction, and spectral display totaled less than 1 min. Results obtained from an equal molar mixture of bovine and porcine insulins are displayed in Fig. 2*E*. These molecules have identical B chains but differ by two amino acid substitutions in the A chain. Signals for the $(M+H)^+$ and doubly charged, $(M+2H)^{2+}$, ions are observed for each molecule.

Fig. 2*F* shows the Fourier-transform mass spectrum of horse cytochrome *c* at the 250-pmol level. Similar data are also obtained for human proinsulin of molecular mass 9387. These results clearly indicate that an analytically significant fraction of large $(M+H)^+$ ions desorbed from the liquid matrix possess internal energies that allow them to survive intact for at least 10 msec.

Resolution and signal/noise ratios demonstrated in Fig. 2 *B–F* are presently limited by our inability (*i*) to monitor the image current or transient from large oligopeptide ions for more than a few milliseconds at a time and (*ii*) to accumulate large organic ions in the cell from more than one ionization event under the new operating conditions. Ions enter the cell along the *z* axis, the direction parallel to the magnetic field, with a total kinetic energy equal to the +1.5 V applied to the probe plus additional energy acquired during desorption from the liquid matrix (13). Following the ionization event, the potential on trapping plate 1 is raised to the experimentally optimized value of +3 V, the same potential as that on trapping plate 2. At this time, ions located on the *z* axis should have kinetic energies slightly greater than +3 V and, therefore, exit the cell through either end plate. For ions to become trapped their kinetic energy in the *z* direction must be reduced below +3 V, presumably by redirecting their motion into the *x–y* plane where ions are confined by the 7-T magnetic field.

Ion image currents for large ion clusters of CsI can be monitored in the present cell for several tens of seconds. Consequently, a resolution in excess of 30,000 for $(CsI)_{22}Cs^+$ at m/z 5848.7 is easily achieved. Because the total ion current produced from a solid sample of CsI exceeds that from the organics by several orders of magnitude, ion–ion repulsion, or space–charge effects, probably account for the efficient redirection of motion into the *x–y* plane for these large cluster ions. The much lower trapping efficiency observed for large organic ions probably results from local field inhomogeneity created by magnetic components employed in the cell construction and by injection of ions into the cell at a slight angle to the *z* axis. An increase in the trapping efficiency of large oligopeptide ions in the cell should result from efforts to reshape the magnetic field, to optimize ion injection, to couple ion injection with ion excitation, and to implement additional electrostatic trapping schemes.

We are indebted to O. P. Tanner and Gary Mappas (Monsanto) and to Michael Story (Finnigan-MAT) for their encouragement and advice. This research was supported by grants awarded to D.F.H., J.S., and D.H.R. from the National Science Foundation and by a gift to D.F.H. from Monsanto.

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