Matrix-assisted Laser-desorption Mass Spectrometry Using 355 nm Radiation

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Matrix-assisted laser desorption of proteins with the 355 nm frequency-tripled output of a neodymium:yttrium-aluminium-garnet laser has been demonstrated. In order to produce desorption at this wavelength, the cinnamic acid derivatives ferulic, caffeic and sinapinic acids have been employed as matrices. Excellent sensitivity (routinely one pmol of protein) and good mass resolution ($m/\Delta m$ 400, Full width at half maximum) have been obtained by this new method. Ultraviolet photo-spectrometry of the cinnamic acid derivative matrices suggests strongly that they can be used as protein laser-desorption matrices at any wavelength between 260 nm and 360 nm, allowing the development of laser-desorption mass spectrometers based on nitrogen or excimer lasers.

The development of the matrix-assisted laser-desorption technique by Karas and Hillenkamp[1-4] has revolutionized the capability of time-of-flight mass spectrometry for the determination of the molecular weight of proteins. Proteins and protein subunits can now have their molecular weights determined with as little as one pmol of sample. This method and the complementary technique of electrospray ionization[5,6] coupled to a quadrupole mass spectrometer, promise to change the way that protein chemists categorize new molecules, adding accurate molecular weight to sodium dodecylsulfate/polyacrylamide gel electrophoresis (better known by the acronymn SDS-PAGE) mobility and amino acid composition data.

In the original laser-desorption technique,[1] the sample protein was mixed into a solution containing a large molar excess of nicotinic acid and this solution was dried on a metal substrate. The deposit was then placed in a time-of-flight mass spectrometer and analyzed by laser desorption, using the pulsed (10 ns) 266 nm frequency-quadrupled output of a neodymium:yttrium-aluminium-garnet (Nd:YAG(4)) laser to produce the desorption. The matrix material (nicotinic acid) absorbs strongly at this wavelength and therefore absorbs the laser radiation preferentially, as compared to the protein embedded in the matrix. A laser pulse with sufficient irradiance causes the mixture of matrix and protein to go into the gas phase and also produces a large number of single or multiply charged ions. Little or no fragmentation has been observed in the spectra obtained by this method.

As originally described, the mass resolution of the technique was approximately $m/\Delta m$ 50 (Full width at half maximum) (FWHM). In recent publications[7,8] we have demonstrated that mass resolutions of greater than 400 can be obtained routinely by careful sample preparation, laser-intensity control and the use of matrices that do not produce strong photochemically induced matrix adduct molecule-ions in the quasimolecular-ion signal. These publications also present a survey of more than fifty compounds as possible matrices for the laser desorption of proteins at $\lambda = 266$ nm.

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In this communication, we report the successful application of pulsed 355 nm laser light (the frequency-tripled output of a Nd:YAG laser, i.e., Nd:YAG(3)) to the desorption of proteins. The applicability of 355 nm is dependent on the use of a different class of matrices that absorb strongly at that wavelength, namely the cinnamic acid derivatives ferulic acid, caffeic acid and sinapinic acid. The properties of these new matrices in reducing matrix adduct-ion formation at 266 nm has been described.[8] Employing these matrix materials and 355 nm radiation, excellent protein mass spectra have been obtained. The sensitivity of the technique with either the Nd:YAG(3) or the Nd:YAG(4) wavelength is quantitatively the same, i.e., typically less than one pmol. This discovery demonstrates clearly that a nitrogen laser ($\lambda = 337$ nm) is a viable alternative to a Nd:YAG laser in a laser-desorption mass spectrometer. The use of this longer wavelength also eliminates any possible absorption of the laser light by the analyte biopolymer, as most classes of biopolymers do not absorb light at this wavelength.

EXPERIMENTAL

Instrumental

The time-of-flight mass spectrometer used was built at Rockefeller University and has been described previously.[7] A schematic plan of the instrument and the data system has also been described in the previous paper.[8] The laser used is a Nd:YAG Q-switched pulsed laser Model Hy-400, (Lumonics Inc., Kanata, Ontario, Canada) including the options for frequency doubling, tripling and quadrupling. The laser has been modified by the removal of the amplifier rod and the addition of a 2 mm diameter mode-selection aperture in the oscillator cavity, mounted on the oscillator shutter assembly. To use the frequency-tripling option, the 1064/526 nm mixing crystal assembly was rotated into the beam and the gull-wing beam separator adjusted so that the output beam contained only 355 nm light. A difference between the current arrangement and the one previously described is that the pyroelectric detector used to generate the "start" pulse has been replaced by a phototransistor.

CCC-0951-4198/89/0436-0439 $2.00

Ultraviolet absorption photo-spectrometry

The ultraviolet absorption spectrum of a matrix material for laser desorption is an important parameter. In order to measure the solid-phase absorption spectrum of a compound being tested as a possible matrix, a thin layer of the material was deposited on the outside of a fused silica cuvette by drying from solution. The cuvette was then placed into a Model Lambda 17 UV/Visible Spectrophotometer, (Perkin Elmer Corp., Edison, NJ, USA) and the absorption spectrum of the layer measured. Although this method is not the most elegant method of obtaining an absorption spectrum from a solid, it does closely resemble the conditions in the laser desorption experiment itself. The absorption spectra obtained allowed the comparison of the relative absorption of a matrix material in the range of 250 nm to 400 nm.

Sample preparation

The matrices employed for protein desorption at 355 nm were 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 3,4-dihydroxycinnamic acid (caffeic acid) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Aldrich Chemical Co., Milwaukee, WI, USA). These materials were dissolved in either a 1:1 mixture of ethanol + 0.1% trifluroacetic acid (TFA) or a 3:2 mixture of 0.1% TFA + acetonitrile to a final concentration of 10 g/L. These solutions were nearly saturated at room temperature. The protein to be examined was dissolved in either 0.1% TFA or a mixture of 0.1% TFA + acetonitrile to a final concentration of 1–5 μM.

To make a sample/matrix deposit, 1 μL of the protein solution was mixed with 5–10 μL of the matrix solution, either by agitation or by immersion in an ultrasonic bath. A small amount, typically 0.5–1.0 μL, of this final solution was placed on the sample probe tip (2 mm diameter, platinum). The volatile solvents were then removed by room temperature evaporation, either by still or forced air, or by vacuum evaporation.

The cinnamic acid derivatives used as matrices are light-sensitive in solution and should be stored in the dark. Prolonged agitation of the solutions in an ultrasonic bath should also be avoided. Matrix solutions were freshly made daily and stored in the dark between use.

Mass spectra

In order to obtain the mass resolution ($m/\Delta m$ 300–500, FWHM) shown, the most important operational parameter was the careful regulation of laser irradiance on the sample. The irradiance per shot was kept as low as possible (close to the threshold of ion production) by use of a variable attenuator.[7] To obtain good output power stability from the laser, the device was operated with a continuous triggering frequency of 5 Hz and on every second pumping-lamp-flash, the oscillator was Q-switched with the internal Pockels cell (i.e., giving an output frequency of 2.5 Hz). The spectra shown were obtained by adding together the recorded detector transients of between 50 and 200 shots. Laser irradiances used were approximately 1 MW/cm^2, averaged over a 100 × 300 μm laser focus.[7] The acceleration voltage (V_{acc}) listed with each spectrum refers to the initial acceleration in the ion source. This acceleration voltage was applied in two stages, in order to reduce the electric field strength near the sample surface.[7] A further 2 kV of post-acceleration was added at the detector.

The spectra shown below have time-of-flight (TOF) as the abscissa, in units of 5 ns and intensity as the ordinate, in arbitrary units, dependent on the number of individual shots summed and the amplifier gain. These plots show the data as collected from the transient recorder, with the indicated number of shots directly summed. In order to improve the S/N ratios of the spectra shown, adjacent channels have been added together to form "bins" corresponding to larger time divisions, as indicated by the parameter "displayed bin size". For example, a "displayed bin size" of twenty ns means that for each data point displayed, four adjacent, five-ns data points have been added together. No background subtraction has been performed.

RESULTS AND DISCUSSION

An important initial result was that of the over fifty matrix materials surveyed at 355 nm, only the three cinnamic acid derivatives (ferulic, caffeic and sinapinic acids) produced any observable protein quasimolecular-ion signals at all. Nicotinic acid, the matrix originated by Hillenkamp and Karas,[1] does not produce a signal. This result was not a surprise, however, because absorption photospectrometry of nicotinic acid shows that it does not absorb light at $\lambda =$ 355 nm. The same holds true for the other matrices so far described, i.e., pyrazinoic acid, thymine and vanillic acid. None of these matrices is useful at this longer wavelength.

The results obtained with the Nd:YAG(3) output for the cinnamic-acid-derivative matrices with proteins were qualitatively the same as those observed using the Nd:YAG(4) output. Figure 1 is a comparison of the ions produced from a sample of horse skeletal apomyoglobin (Mol. Wt 16950.5) in a matrix of ferulic acid at: (a) $\lambda = 266$; and (b) $\lambda = 355$ nm. From these spectra and many other examples, it is quite clear that the spectra obtained from the tripled frequency contain as much information and are of the same quality as those obtained from the quadrupled frequency. The sensitivity is also very similar in both cases, being typically less than one pmol for the proteins examined.

The capabilities of this technique for measuring the molecular weights of proteins with both accuracy and sensitivity can be demonstrated using the example of a well-known protein, ribonuclease S. Ribonuclease S is produced by the action of the endoprotease subtilisin (from *Bacillus subtilis*) on pancreatic ribonuclease A.[9] This protease cleaves the protein at the Ala(20)-Ser(21) bond to form two separate chains, usually referred to as the S-peptide (residues 1–20) and the S-protein (residues 21–124).[10] The two chains are held together strongly by solvation forces and after the cleavage still function in exactly the same fashion as pancreatic ribonuclease A. Figure 2 shows the S-protein quasimolecular-ion region in the spectrum of bovine pancreatic ribonuclease S (Sigma, cat. no. R6000). The spectrum shows that the material contains two major components. The measured molecular weight of the species M (11 533.0) corresponds closely to the calculated molecular weight of the S-protein (Mol. wt

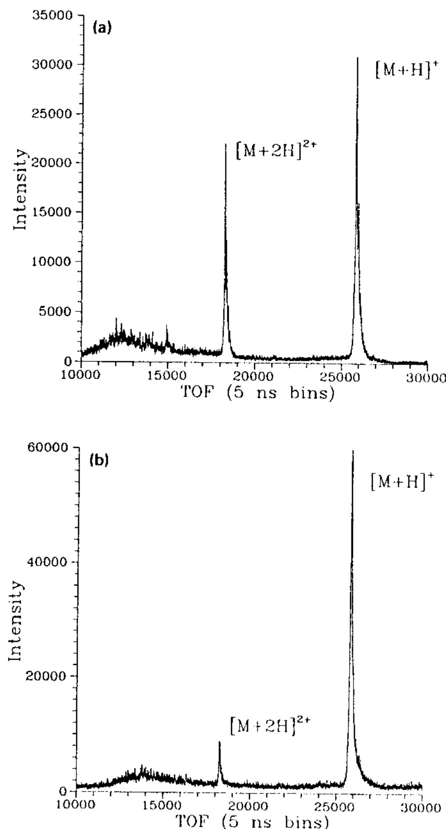

Figure 1. The positive-ion laser-desorption time-of-flight mass spectra of equine skeletal apomyoglobin (mol. wt 16950.5) using ferulic acid matrix and comparing different desorption laser-output wavelengths: (a) Nd:YAG(4) ($\lambda = 266$ nm); and (b) Nd:YAG(3) ($\lambda = 355$ nm). Conditions: V_{acc} 20 kV; 200 laser shots summed; total protein loaded 1 pmol; displayed bin size = 20 ns.

11 533.9). The measured molecular weight of the species M′ (11 446.6) corresponds to the calculated molecular weight of a known S-protein variant produced by the cleavage of the Ser(21)-Ser(22) bond (11 446.9).[11,12] In order to determine the quantity of this additional protein in a preparation of ribonuclease S by standard methods, at least four cycles of quantitative Edman degradation analysis must be performed. The same result can be obtained by laser-desorption mass spectrometry with a small fraction of the effort. The two

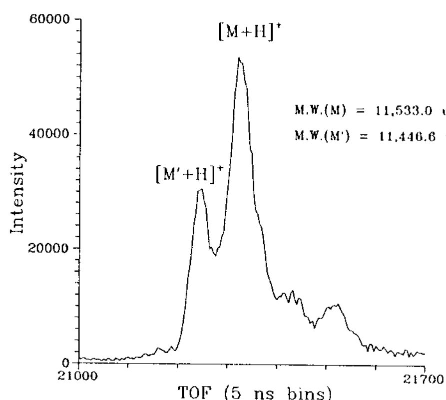

Figure 2. The quasimolecular-ion region of a positive-ion laser-desorption time-of-flight mass spectrum of pancreatic ribonuclease S S-protein (see text) using a sinapinic acid matrix. Conditions: V_{acc} 20 kV; 200 laser shots summed; total protein loaded 3 pmol; displayed bin size 20 ns.

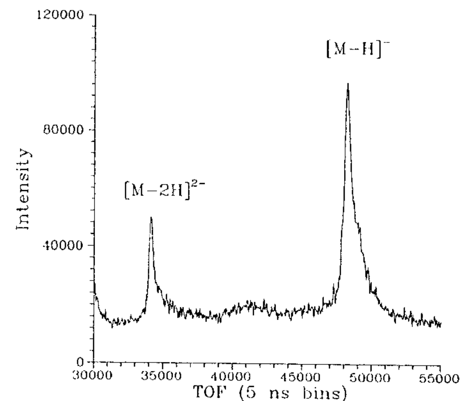

Figure 3. The negative-ion laser-desorption time-of-flight mass spectrum of covalently cross-linked tropomyosin dimer (measured mol. wt 65,472, calculated mol. wt 65,476[11]) using a sinapinic acid matrix. Conditions: V_{acc} −22.5 kV; total protein loaded 0.5 pmol; displayed bin size 200 ns.

high mass shoulders on the right of Fig. 3 are caused by photochemically generated adducts of the matrix to the proteins.[8]

It was found previously[7] that negative ions were produced from matrix-assisted protein laser-desorption using Nd:YAG(4). The same finding holds true for 355 nm. Figure 3 shows the mass spectrum of the covalently cross-linked rabbit skeletal muscle tropomyosin dimer. Tropomyosin normally exists as a straight single chain,[13] but can be covalently cross-linked easily to form a dimer because of a single, unpaired cysteine residue in the chain. The spectrum was produced from 0.5 pmol of protein loaded onto the sample tip, demonstrating that the sensitivity of the negative-ion mode is at least comparable with the positive-ion mode.

The discovery that a laser using a wavelength much longer than that of the Nd:YAG(4) output produces useful spectra opens up the possibility of using other types of lasers for laser-desorption mass spectrometry. In addition to the Nd:YAG(3) output, some lasers that may be useful are: (i) a nitrogen laser ($\lambda = 337$ nm); (ii) an excimer laser, operating either with XeCl ($\lambda = 308$) or XeF ($\lambda = 350$ nm); (iii) an argon laser ($\lambda = 351$ or 364 nm); or (iv) a frequency-doubled ruby laser ($\lambda = 347$ nm). The absorption spectra of sinapinic, ferulic and caffeic acids (see the Experimental section) show no strong minima between 260 nm and 360 nm suggesting that these laser types should work almost interchangeably, as long as an equivalent irradiance can be produced on the sample, i.e., approximately 1 MW/cm^2. It should be noted that the Nd:YAG laser used in our work produces a very high quality, low divergence (<0.5 milliradians) output beam profile, that allows the use of a very simple optical system. Gas lasers with short oscillator cavities and high beam divergences will probably require a more elaborate optical assembly to produce the same power density on the sample.

Acknowledgements

This work was supported by grants RR 00862, GM 38274 and BRSG S07 RR07065 from the US National Institutes of Health. We would also like to thank Dr. B. Manjula (Rockefeller University) for supplying us with the samples of tropomyosin and ribonuclease S.

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Received 23 October 1989; accepted 29 October 1989.