

# Mass spectrometric molecular-weight determination of highly acidic compounds of biological significance via their complexes with basic polypeptides

(matrix-assisted laser desorption/mass spectrometry/oligonucleotides/heparin/suramin)

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**ABSTRACT** Highly acidic compounds that are difficult to ionize by matrix-assisted laser desorption ionization give excellent spectra when mixed with a basic peptide or protein to form a noncovalent complex. This phenomenon makes it possible to determine the molecular weights of polysulfated, -sulfonated, and -phosphorylated biomolecules such as cysteine acid-containing peptides, oligonucleotides, heparin-derived oligosaccharides, and suramin (a drug containing two trisulfonated naphthalene moieties). Peptides and small proteins rich in arginine were used as the basic components. The extent of complex formation correlates with the number of phosphate and sulfate groups in the acidic component and with the number of arginines in the basic component. Neither the acidic amino acid residue aspartic and glutamic acid nor the basic lysine and histidine contribute to complex formation. For oligonucleotides, histone H4 was found to be the best complexing agent investigated. The analytical utility of the complex formation is demonstrated by the molecular-mass determination of acidic compounds from 500 to 6000 Da at the picomole or sub-picomole level with an accuracy of  $\pm 0.1\%$  or better and by the absence of alkali cation adducts.

The development of matrix-assisted laser desorption ionization (MALDI) mass spectrometry permits the determination of the molecular mass of proteins up to the  $10^5$ -Da range with an accuracy of 0.1–0.01%, requiring only picomoles or sub-picomoles of material (1–4). The method is equally applicable to smaller biologically important molecules, such as peptides (5), carbohydrates (6), oligonucleotides (7, 8), glycolipids (9), and polar and nonpolar synthetic polymers (10, 11). It has become an important technique in biochemistry and biology, not only because the molecular weight of the native material at that level of accuracy is in itself very useful information, but also because the changes thereof upon chemical or enzymatic treatment provide further insight into the structure or biological significance of parts of the native molecule (12). These manipulations are often necessary to obtain structural information because little excess energy is transferred to the analyte during the MALDI process, and “prompt” fragmentation is therefore rarely observed. This feature is an advantage in the analyses of mixtures, as long as the components can be resolved.

Although most of the compounds in the above-mentioned categories are amenable to MALDI, it is difficult to ionize highly acidic compounds, even in the negative mode of the mass spectrometer, where they are detected as anions. In that mode, most efforts have been devoted to oligonucleotides (7, 8). It is even more difficult to ionize polysulfate esters or polysulfonic acids. This is due, in part, to the fact that these substances tenaciously attach cations (such as

$\text{Na}^+$ ,  $\text{K}^+$ , etc.) that give rise to broad unresolved peaks, the centroid of which corresponds to the average mass of all these partial salts.

When attempting to use the oxidized A chain of bovine insulin ( $A_{\text{ox}}$ , a 21-amino acid peptide containing four cysteine acid residues) as the internal standard to establish the accuracy of the measurement of the molecular weights of small proteins, like bovine insulin (in the positive-ion mode and with sinapinic acid as the matrix), we observed an unexpected phenomenon: there was no signal at all for  $A_{\text{ox}}$ ,  $[M_{A_{\text{ox}}} + \text{H}]^+ = m/z 2532.7$  (where  $M$  represents the mass of the molecule), but there was a strong signal for its protonated 1:1 complex with intact insulin (ins)  $\{[M_{\text{ins}} + M_{A_{\text{ox}}} + \text{H}]^+, m/z 8264.9$  (found),  $m/z 8266.3$  (calculated)} in addition to a peak of  $[M_{\text{ins}} + \text{H}]^+$ ,  $m/z 5734.5$ . This finding has two consequences: (i) Care must be taken when analyzing mixtures of peptides or proteins containing highly acidic components. They may be missed, and other peaks may appear at an  $m/z$  value that corresponds to a complex of two of the components, rather than the  $(M + \text{H})^+$  ion of an individual compound. (ii) More importantly, it should be possible to obtain excellent MALDI mass spectra in the positive-ion mode for highly acidic (or highly basic) compounds that are otherwise extremely difficult to ionize. A detailed evaluation of this second and more important part of the original observation is the subject of the work described below.

## MATERIALS AND METHODS

The MALDI experiments were done on a modified VT2000 (Vestec, Houston) linear time-of-flight mass spectrometer previously described (13). Two laser sources were used in this study: a  $\text{N}_2$  laser radiating at 337-nm wavelength with 3-ns pulses (Laser Science, Newton, MA) for ultraviolet experiments (UV-MALDI), and an erbium:yttrium/aluminum-garnet laser (Schwartz ElectroOptics, Orlando, FL) with 2.94- $\mu\text{m}$  wavelength and 120- to 140-ns pulses for infrared experiments (IR-MALDI).

The ions generated by the laser pulses were accelerated typically to 30 keV ( $1 \text{ eV} = 1.602 \times 10^{-19} \text{ J}$ ) energy. Recently, a stainless-steel electrostatic particle guide (0.5-mm diameter) has been installed along the axis of the drift tube to improve ion transmission (14). The guide wire was appropriately pulsed to protect the detector from overload due to the abundant low-mass matrix ions. As reported by Brown and Gilfrich (15), the use of the particle guide not only increased sensitivity but also increased the mass resolution. Under

Abbreviations: MALDI, matrix-assisted laser desorption ionization;  $A_{\text{ox}}$ , oxidized A chain of bovine insulin;  $M_A$ , mass of acidic molecule;  $M_B$ , mass of basic molecule; TPKS, tyrosine protein kinase substrate.

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optimum conditions, a resolution of 1000 (at full width at half-maximum) was obtained at  $m/z$  5734.5 (bovine insulin).

Ions were detected with a 20-stage discrete dynode electron multiplier or with a hybrid detector consisting of a microchannel plate and a discrete dynode electron multiplier. The detector signal was preamplified and digitized by a digitizing oscilloscope (LeCroy, Chestnut Ridge, NY) at a rate of 400 or 200 MHz, depending on the time-of-flight range covered by the measurement. The software for data acquisition and processing developed in our laboratory runs on an IBM personal computer and a Local Area VAXcluster. The software is flexible enough to program the oscilloscope for automatic averaging of a number of individual mass spectra or for interactive averaging allowing the operator to include a spectrum into the average or to discard it on a one-by-one basis. The former operation is well suited to UV-MALDI experiments, where the shot-to-shot variation of the mass spectra is reasonably low. Interactive averaging, however, is a necessity with IR-MALDI, where considerable shot-to-shot variation of the mass spectra and the higher consumption rate of the sample (16) require an economic method of data acquisition.

For MALDI, the analyte has to be embedded in a large excess of well-absorbing matrix molecules—mostly small, solid organic acids. Over 20 matrix compounds have been tested in the complex formation experiments. The most efficient matrices were sinapinic acid, caffeic acid, anthranilic acid in the UV, and succinic acid and 5-(trifluoromethyl)uracil in the IR. All the matrix compounds were purchased from Aldrich and used without further purification. Matrix solutions were prepared at  $\approx 10$  g/liter concentration in caffeic acid/5-(trifluoromethyl)uracil, 1:1, or sinapinic acid/anthranilic acid, 2:1, water/acetonitrile solvent or in pure water (succinic acid).

Synthetic peptides were prepared in the Biopolymer Laboratory at the Massachusetts Institute of Technology, other test peptides (Table 1) were purchased from Sigma, peptide SP-3 was provided by T. Curran (Roche Institute of Molecular Biology, Nutley, NJ), and histone H4 from calf thymus was purchased from Boehringer Mannheim and used without further purification. Phosphated and sulfated compounds were used as the acidic components. Oligonucleotides were synthesized at the Massachusetts Institute of Technology, a heparin-derived hexasaccharide was obtained from D. J. Tyrrell (Glycomed, Alameda, CA), and suramin was provided by W. C. Herlihy (Glycan Pharmaceuticals, Cambridge, MA). The sulfated compounds were provided as sodium or ammonium salts. Initially, cation-exchanger beads (AG 50W-X8, Bio-Rad) were used to convert the salts into free acids. This procedure was found unnecessary, however, and the salts were used in the later experiments. In most cases, the basic and acidic components were mixed in 1:1 molar ratio and diluted with the matrix solution. The final analyte concentrations were between 0.1 and 10 pmol/ $\mu$ l. A volume of 0.5 to 1  $\mu$ l of the final solution was placed on the probe tip and dried with the assistance of an airstream.

## RESULTS AND DISCUSSION

**The Phenomenon.** Protonated dimers, trimers, etc., of the analyte are often observed in MALDI mass spectra in decreased abundance with increased multiplicity. In contrast, we have found that when analyzing mixtures of strongly acidic and basic biopolymers, ionic complexes often account for the most abundant signals. The variation of the abundance of these complex ions is not necessarily monotonic, and the signals for one or both uncomplexed component(s) may even be absent. In the following discussion, complex ions are denoted  $[mM_B + nM_A + H]^+$ , where  $M_B$  and  $M_A$  refer to the molecular weight of the basic and acidic components, re-

spectively, and  $m$  and  $n$  refer to their multiplicity. For the sake of brevity,  $(m:n)^+$  will be used to describe composition and charge state of the complex—for example,  $(1:0)^+$  for  $[M_B + H]^+$ ,  $(1:1)^+$  for  $[M_B + M_A + H]^+$ ;  $(1:2)^+$  for  $[M_B + 2M_A + H]^+$ ;  $(1:1)^{2+}$  for  $[M_B + M_A + 2H]^{2+}$ , etc.

An illustrative example is shown in Fig. 1, the IR-MALDI mass spectrum of an equimolar mixture of the  $A_{ox}$  and tyrosine protein kinase substrate (TPKS,  $M_r = 1592.7$ ) in succinic acid as the matrix. The most abundant ion corresponds to the protonated 1:1 complex,  $(1:1)^+$ , but a number of higher oligomers,  $(1:2)^+$ ,  $(2:1)^+$ ,  $(2:2)^+$ , and  $(2:3)^+$  are also observed. Although the signal for the acidic component,  $A_{ox}$ ,  $(0:1)^+$  is low and very broad due to extensive alkali ion attachment, the singly and doubly protonated ions of TPKS,  $(1:0)^+$  and  $(1:0)^{2+}$  formed sharp peaks and were used for internal calibration of the mass scale. For the  $(1:1)^+$  complex ion  $m/z$  4124.2 was obtained in excellent agreement with the calculated value of 4125.4 (that is, the sum of the components).

Ionic complexes could be observed upon UV or IR irradiation. Their abundance in MALDI mass spectra depends on three parameters: the basic component, the acidic component, and the matrix. The effectiveness of the basic components was evaluated based on the relative abundance ratio  $(1:1)^+/(1:0)^+$  for  $A_{ox}$  as the acidic component and sinapinic acid as the matrix. Basic peptides and proteins included in this study are compiled in Table 1. In addition to naturally occurring polypeptides, several synthetic peptides of high arginine content have also been tested. The data (not shown) obtained with TPKS, renin substrate-(1–13), and  $\beta$ -endorphin indicate that complex formation [i.e., relatively more abundant  $(1:1)^+$  ion] appears to depend on increased numbers of arginines but is not affected by the number of the less basic lysines and histidines present. For example, no complex of  $A_{ox}$  was observed with  $\beta$ -endorphin, which contains five lysines but no arginine. The significance of the number of arginines is also related to the size of (and arginine distribution within) the protein: moderate complexing between histone H4 and  $A_{ox}$  was observed, whereas the less basic growth hormone-releasing factor was more effective. For larger peptides and proteins, their tertiary structure seems to play a significant part.

The number of acidic sites and their  $pK_a$  value are equally important for the acidic counterpart  $M_A$ . Whereas the  $A_{ox}$  complexes readily, the oxidized B chain (two cysteic acids within the 30-amino acid peptide,  $M_r = 3495.9$ ) produces hardly any complex ions, and pancreastatin-(37–52) with five glutamic acids located at the N terminus of this hexadecapeptide ( $M_r = 1820.0$ ) forms no complexes at all, even with the most basic peptides. Complex formation is most important

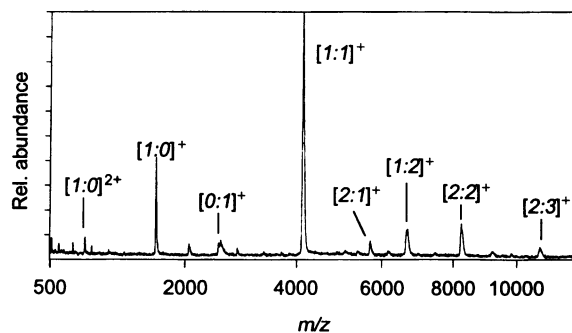


FIG. 1. IR-MALDI mass spectrum of an equimolar mixture of TPKS ( $M_r = 1592.7$ ) and  $A_{ox}$  ( $M_r = 2531.7$ ) (matrix, succinic acid). The spectrum is an average of 20 laser shots. Although accurate mass measurement could not be accomplished on the  $(0:1)^+$  ion, excellent accuracy (2530.5 Da) was obtained from the  $(1:1)^+$  ion of  $m/z$  4124.2. Rel., relative.

Table 1. Basic components used in the complex formation experiments

Basic component	Sequence	$M_r$
Neurotensin-(8-13)	RRPYIL	818.01
Dynorphin-(1-9)	YGGFLRRIR	1137.36
Synthetic peptide SP-1	RKKRRQRRR	1339.62
Synthetic peptide SP-2	RRRRRRPYIL	1441.76
Tyrosine protein kinase substrate (TPKS)	RRLIEDNEYTARG	1592.74
Renin substrate-(1-13)	DRVIYHPFHLVIH	1645.92
Melittin	GIGAVLKVLTTLG-PALISWIKRKRQQ	2847.49
Synthetic peptide SP-3	IRRERNKMAAAK-SRNRRELDTL	2942.41
$\beta$ -Endorphin	YGGFMTSEKSQTP-LVTLFKNAIKNA-YKKGE	3465.04
Growth hormone releasing factor (bovine)	YADAIFTNSYRKV-LGQLSARKLLQDI-MNRQQGERNQE-QGAKVRL	5108.83
Insulin (bovine)	Arginine, 1; lysine, 1; histidine, 2	5733.56
Ubiquitin (bovine)	Arginine, 4; lysine, 11; histidine, 2	8564.85
Histone H4 (calf thymus)	Arginine, 14; lysine, 11; histidine, 2	11236.2*
Cytochrome <i>c</i> (horse)	Arginine, 2; lysine, 18; histidine, 3	12360.1

\* $M_r$  based on the amino acid sequence without posttranslational modifications.

with highly sulfated, sulfonated, and phosphorylated compounds.

The effect of the matrix is also important. The MALDI mass spectrum of an equimolar mixture of bovine ubiquitin and  $A_{ox}$  is shown in Fig. 2A for sinapinic acid and in Fig. 2B for  $\alpha$ -cyano-4-hydroxycinnamic acid as the matrices. The latter spectrum is dominated by signals for the singly, doubly, and triply charged ubiquitin, but the signal for  $A_{ox}$  is entirely absent (see arrow), and only a little of its complex with the

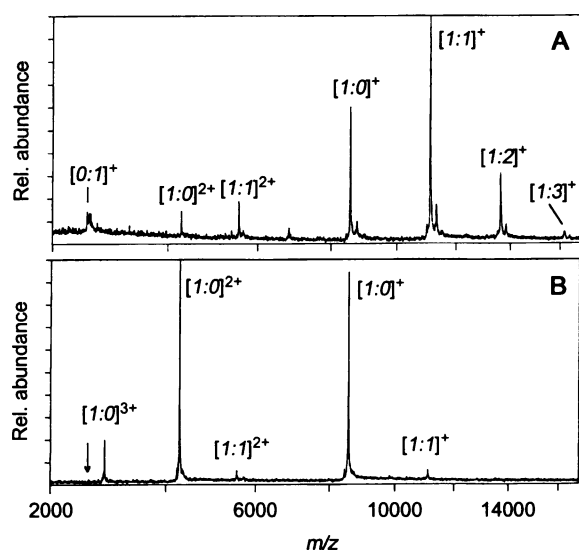


FIG. 2. MALDI mass spectra of an equimolar mixture of bovine ubiquitin ( $M_r = 8564.9$ ) and  $A_{ox}$  (wavelength: 337 nm). (A) Matrix, sinapinic acid; the small satellite peaks visible for the more abundant ions are photoadducts of the matrix. (B) Matrix,  $\alpha$ -cyano-4-hydroxycinnamic acid; the arrow points to the position where the  $[M + H]^+$  ion of  $A_{ox}$  would be expected. Rel., relative.

protein is observed. However, with sinapinic acid as the matrix (Fig. 2A), the most prominent peak is due to the  $(1:1)^+$  complex, in addition to major peaks representing the  $[M + H]^+$  ion of ubiquitin itself  $(1:0)^+$  and its protonated complex with two  $A_{ox}$  molecules  $(1:2)^+$ . There is a very small signal for  $A_{ox}$ , broadened by alkali ion adducts.

**Application to Oligonucleotides.** Small oligodeoxyribonucleotides (< 10-mers) formed complexes with many of the basic peptides listed in Table 1. However, the abundance of the complex ions was considerably lower than those with  $A_{ox}$ , indicating a weaker interaction of the phosphate groups with the arginines than was observed with sulfonic acids. As matrices, sinapinic acid, anthranilic acid, and 3-aminopyrazine-2-carboxylic acid were most effective in the formation of complex ions.

Larger oligonucleotides did not form complexes with the smaller peptides, perhaps because the higher order of structure of the nucleotides interferes with the stabilization of the complex. Because histones are some of the strongest DNA-binding proteins (17) and histone H4 has the highest arginine content among the inner histones (18), its suitability as a complexing agent was explored.

The UV-MALDI mass spectrum of an equimolar mixture of H4 and single-stranded  $d[T]_{10}$  (Fig. 3) exhibits abundant  $(1:1)^+$  and a low level of  $(1:2)^+$  complex ions. The peak for the protonated histone,  $(1:0)^+$ , centers around  $m/z$  11387, and the  $(1:1)^+$  complex is found at  $m/z$  14316. The difference of 2929 is somewhat lower than 2980.0, the  $M_r$  of  $d[T]_{10}$ . It is of interest to note that the peak of the complex ion is narrower ( $\Delta M = 220$  Da at full-width at half-maximum) and more symmetrical than that of the H4 ion, which has a  $\Delta M$  of 310 Da. The broadness of the latter peak is partly due to the inhomogeneity of the posttranslational modifications of H4 (five acetylation and two methylation sites) (18) and may also be due to the attachment of inorganic anions, which could cause the trailing high-mass side of the peak. The narrower complex peak could be explained by the displacement of the anions by the nucleotide or selective complexing of the less acetylated components of H4. The latter possibility is less likely because the acetylation involves the N terminus and the four lysines nearby, and as we have already mentioned, even unacetylated (i.e., still basic) lysine has little complexing effect. MALDI mass spectrum of a larger oligonucleotide,  $dp[T]_{20}$ , with histone H4 also showed the  $(1:1)^+$  ion, but the signal was considerably lower. These experiments demonstrate that the complexing phenomenon is applicable to oligonucleotides, but to obtain accurate molecular weight information a homogeneous arginine-rich polypeptide must be used.

**Application to Heparin-Derived Oligosaccharides.** The glycosaminoglycan heparin is a linear, polydisperse, highly sulfated polysaccharide ranging in molecular mass from 5 to 40 kDa. It is a very heterogeneous polymer composed of

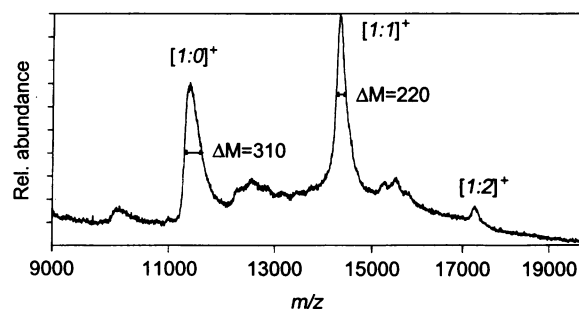
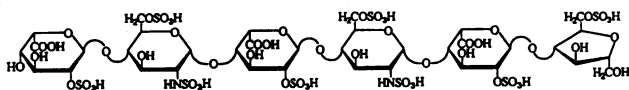


FIG. 3. UV-MALDI mass spectrum of the complex of histone H4 from calf thymus  $[(1:0)^+ = m/z 11387]$  obtained by external calibration with decahydridic acid  $d[T]_{10}$  ( $M_r = 2980.0$ ) (matrix, sinapinic acid). Rel., relative.

disaccharide units, which consist of a uronic acid (D-glucuronic or L-iduronic acid) and a glucosamine, which are sulfated to various degrees on the -OH and -NH<sub>2</sub> groups; the latter are always either acetylated or sulfated. In addition to its long-standing and wide use as an anticoagulant, heparin has many other biological functions, but its detailed structure is undefined (19). These polymers can be degraded enzymatically and/or with nitrous acid into smaller subunits more amenable to structure determination. Because of their heterogeneity, obtaining the molecular weights of these components is an important first step.

Mass spectrometric investigation of heparin-derived oligosaccharides poses a serious challenge because they have to be extensively purified and desalted for negative-ion fast-atom-bombardment mass spectrometry. This methodology requires large amounts of material (10 nmol/ $\mu$ l) and still results in partially sodiated anions of monosulfated disaccharides and polysulfated di- to octasaccharides, which contain up to 15 Na<sup>+</sup> ions (20–22). Probably because of these difficulties, little mass spectrometric work concerning this biologically important class of compounds has been reported to date.

Similar difficulties are also encountered with MALDI, in spite of its intrinsically higher sensitivity. For di- and trisulfated heparin-derived disaccharides, as much as 100 pmol/ $\mu$ l was required to obtain a negative-ion signal, and even then the signal-to-noise ratio was poor. However, upon addition of a basic peptide, sub-picomole sensitivity in the positive-ion mode was attained. When the octasulfated hexasaccharide (Structure 1) at 3 pmol/ $\mu$ l (23), was mixed with a basic peptide such as SP-3 (Table 1), the spectrum shown in Fig. 4 resulted.



Structure 1

It exhibits good signals related to the (1:1)<sup>+</sup> ion, but some fragmentation takes place. The most abundant ion (1:1-2SO<sub>3</sub>)<sup>+</sup> (found:  $m/z$  4441.1, calculated 4438.8), accompanied by the complex that has lost one SO<sub>3</sub> group ( $m/z$  4519.2, calculated 4518.8) and three SO<sub>3</sub> groups ( $m/z$  4363.5, calculated 4358.8), respectively. The (1:0)<sup>+</sup> ion was found to be  $m/z$  2943.7 (calculated 2943.4) by external calibration. Because of the structural constraints of nitrous acid degradation products of heparin, at this level of mass accuracy (0.05% for the averaged values of the three signals) the information provided by the mass spectrum allows one to conclude unambiguously that the material is a hexasaccharide with a total of seven or more sulfation sites where all the glucos-

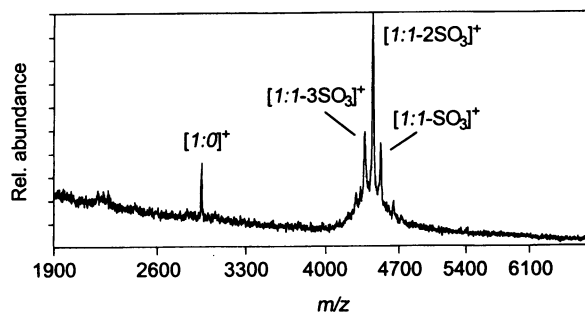
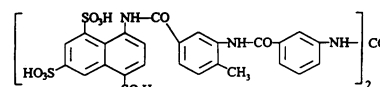


FIG. 4. MALDI mass spectrum of an equimolar mixture (3 pmol each) of the heparin-derived hexasaccharide I with the synthetic peptide SP-3 (wavelength, 337 nm; matrix, sinapinic acid). The small peak at  $m/z$  4650 corresponds to the photoadduct of the matrix on the most abundant ion. Rel., relative.

amine residues are N-sulfated. For the known octasulfated hexasaccharide (I), the  $m/z$  value of the (1:1-SO<sub>3</sub>)<sup>+</sup> ion would give  $M_r = 1655.8$ , whereas the calculated value is 1655.4. It should be noted that no cation adducts were observed for any of the peptide-heparin complexes we have measured, even though the sulfated oligosaccharides were used as sodium or ammonium salts.

Any information that reveals the number of carbohydrate units and the total occupied sulfation sites of compounds obtained by enzymatic or chemical degradation of heparin, and of the less-sulfated heparan, is important for the determination of the overall structure of these polysaccharide chains attached to proteins to form the biologically significant proteoglycans.

**Application to Aromatic Polysulfonic Acids.** Suramin (Structure 2) has been used for many decades as an effective drug against *Trypanosoma* viruses, which cause sleeping sickness and river blindness, and is also a potent inhibitor of the reverse transcriptase activity of retroviruses (24). Again, the high polarity of the two trisulfonic acid moieties makes it difficult to ionize suramin.



Structure 2

For mass spectra produced by fast-atom-bombardment ionization, a signal-to-background ratio of 100 is reported without specifying the amount of material required (probably nanomoles) (25). The MALDI spectrum (data not shown) of the free acid (generated by mixing the sodium salt with a few cation-exchange beads) can be obtained in the negative-ion mode with 2,5-dihydroxybenzoic acid as matrix, but it still exhibits Na<sup>+</sup> adducts. However, upon addition of a basic peptide, abundant complex ions (free of cation adducts) are produced in the positive-ion mode. A typical spectrum obtained with suramin at  $\approx 5$  pmol/ $\mu$ l and a 2-fold molar excess of TPKS with sinapinic acid as the matrix is shown in Fig. 5. Under these conditions, the (2:1)<sup>+</sup> complex gives rise to the most abundant ion, possibly because one peptide molecule each complexes with one of the naphthyltrisulfonic acid moieties. The higher order complexes may be linear aggregates, and the (1:1)<sup>+</sup> and (2:1)<sup>+</sup> complex ions are still detectable at a level of suramin at 0.075 pmol/ $\mu$ l, indicating the remarkable sensitivity of MALDI for highly sulfonated compounds when complexed in this manner.

Strong complex ions were also obtained with mixtures of basic peptides and suramin analogues containing only two sulfonic acid groups on the naphthalene moieties and linked by only two or three aminobenzoic acid units. Thus, the

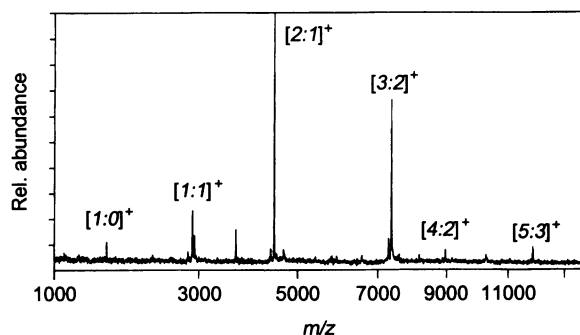


FIG. 5. UV-MALDI mass spectrum of a mixture of suramin ( $M_r = 1297.2$ , free acid) with a 2-fold molar excess of TPKS. Rel., relative.

effect of the complexing with basic components is a general property of this group of naphthylsulfonic acid derivatives.

### CONCLUSION

Efficient complex-ion formation permits the determination of the molecular weights of strongly acidic or strongly basic components that are otherwise difficult or impossible to ionize by MALDI. In the cases reported here, this was always the acidic counterpart. The molecular weight could be measured with high sensitivity (picomoles or less) and good mass accuracy, upon addition of a peptide or small protein of known structure containing a number of arginines. In other words, by using basic peptides and proteins as "carriers" for the acidic components detection sensitivity of the latter reaches the low level usually achieved for peptides and proteins. The results obtained by IR irradiation are similar to those shown for UV-MALDI. From our present results, it appears that the observed phenomenon is most useful for the mass spectrometric analysis of polysulfated compounds, particularly the structural components of heparin and related substances, as well as polysulfonic acids. Complexes are also formed with oligonucleotides. This approach may be further improved by optimizing both the matrix as well as the structure and size of the basic component. Although in the examples described here, the compounds for which molecular weight is to be determined were always the acidic components, the reverse would also be possible.

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