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Ice as a matrix for IR-matrix-assisted laser desorption/ionization: Mass spectra from a protein single crystal

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ABSTRACT **Lasers emitting in the ultraviolet wavelength range of 260–360 nm are almost exclusively used for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) of macromolecules. Reports about the use of lasers emitting in the infrared first appeared in 1990/1991. In contrast to MALDI in the ultraviolet, a very limited number of reports on IR-MALDI have since been published. Several matrices have been identified for infrared MALDI yielding spectra of a quality comparable to those obtained in the ultraviolet. Water (ice) was recognized early as a potential matrix because of its strong O—H stretching mode near 3 μm. Interest in water as matrix derives primarily from the fact that it is the major constituent of most biological tissues. If functional as matrix, it might allow the *in situ* analysis of macromolecular constituents in frozen cell sections without extraction or exchanging the water. We present results that show that IR-MALDI of lyophilized proteins, air dried protein solutions, or protein crystals up to a molecular mass of 30 kDa is possible without the addition of any separate matrix. Samples must be frozen to retain a sufficient fraction of the water of hydration in the vacuum. The limited current sensitivity, requiring at least 10 pmol of protein for a successful analysis needs to be further improved.**

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has become a widely used technique for the analysis of large biological molecules (1). Lasers emitting in the ultraviolet (UV) wavelength range of 260–360 nm and matrices absorbing strongly in this range are almost exclusively used in MALDI instruments. First reports about the use of lasers emitting in the infrared (IR) at wavelengths around 3 μm [erbium-yttrium-aluminium garnet laser] and 10.6 μm (CO_2 laser) appeared as early as 1990 (2, 3). A host of potential matrices are available with a strong absorption at these wavelengths, mainly resulting from O—H and N—H vibrational modes at 3 μm. Several of them, such as succinic acid, glycerol, or urea, have been proven to be functional matrices yielding spectra of a quality comparable to those obtained in the UV (4). The IR absorption, even though a factor of at least 10 weaker than those encountered in the UV, leads to enough deposition of energy into the sample to make plausible the *desorption* of molecules. In addition, the fact that the spectral essential features recorded with either UV or IR wavelengths are virtually identical suggests that the mechanisms of desorption and the function of the matrix are essentially identical in both cases. However, the *ionization* step in IR-MALDI is still unknown, in contrast to the UV for which credible models have been suggested (5). Water in the frozen state (to provide compatibility with the vacuum requirements of the spectrometer) was recognized early as a potential matrix because of its strong O—H stretching mode near 3 μm. Interest in water as matrix derives primarily from the fact that it is the major constituent of most biological tissues and is naturally associated with the vast majority of biological molecules. If functional, it might allow the *in situ* analysis of macromolecular constituents in frozen cell sections without extraction or exchanging the water with another suitable matrix. However, attempts to obtain spectra from samples of bulk, frozen aqueous protein solutions failed (6). Sporadic protein signals of poor signal-to-noise-ratio and reproducibility were obtained exclusively from the very rim of the sample. The latter observation is in agreement with results reported by Williams and coworkers (7, 8) who also used frozen solutions of DNA as well as a few proteins for laser ablation time-of-flight MS. Their experimental approach and rational is, however, very different from the standard MALDI technique. They placed a layer of the frozen sample solution of a few micrometers thickness onto a corroded copper surface and irradiated with a wavelength of 589 nm and an irradiance of about 10^9 W/cm^2, exceeding that of typical MALDI conditions by three orders of magnitude. The laser energy is assumed to be absorbed by the copper substrate; sodium, resonantly excited by the 589-nm photons, is believed to assist in the ionization process. Interestingly, they also encountered the problem of very limited reproducibility and reported that good spectra are obtained from the sample rim only.

This paper reports the rather unexpected and surprising results of a series of systematic experiments designed to assess the utility of ice as a MALDI-matrix in the infrared.

EXPERIMENTAL PROCEDURES

An in-house built reflectron time-of-flight instrument of 3.5 m equivalent flight length and a 12 kV acceleration potential was used for all experiments (3). Working pressure in the sample chamber is 3×10^{-4} Pa. Ions are detected by a conversion dynode (ion impact energy 20–27 kV, depending on ion mass) mounted 1 cm in front of a venetian blind secondary electron multiplier (EMI R2362). Signals are processed by a transient recorder with a time resolution of 10 ns (LeCroy 9400A) and then transferred to a personal computer for storage and further evaluation. Single pulses of an erbium-yttrium-aluminum garnet laser (SEO 1-2-3, Schwartz Electro Optics, Orlando, FL) of ≈150 ns duration at a wavelength of 2.94 μm are focused to a spot diameter of 180 μm with a 45° angle. The instrument is equipped with a liquid nitrogen cooling stage. The temperature of the stainless steel substrate is monitored with a thermocouple with an absolute accuracy of ±5 K, but it may be possible that the actual temperature of the top layer, even of very thin samples, is slightly higher than the measured substrate temperature.

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Abbreviation: MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry.

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RESULTS

All attempts to improve quality and reproducibility of protein spectra obtained from samples of bulk, frozen aqueous solutions (typically 3–10 μl) by variation of all accessible parameters, in particular, the concentration of analyte solution from 10^{-6} M to saturation (which for most biomolecules is about 10^{-4} M–10^{-3} M), source pressure, addition of salts (sodium, potassium), magnitude of irradiance from 2×10^6 W/cm^2 to 5×10^7 W/cm^2, variation of substrate material (copper, stainless steel, silver, aluminum, etc.) gave very unsatisfactory results: either no signal or only those of water clusters up to a mass of ≈5000 Da were obtained. Protein signals of poor signal-to-noise-ratio (typically s/n ≤ 10) were obtained only from the very rim of the sample and only in 10–30% of the exposures, depending on the sample temperature. Fig. 1 shows a spectrum that was obtained from a 3×10^{-3} M solution of lysozyme at T = 160 K. Resolution of the M^+ is only ≈40 full width at half maximum (FWHM) with the doubly charged lysozyme as the base peak.

The situation changes dramatically if the samples (typically 3–5 μl of a 10^{-3} M solution) are first "dried" under ambient conditions before being frozen to liquid nitrogen temperature. Similar results are obtained if a few flakes of lyophilized protein are applied directly to the substrate and frozen. All samples were frozen by dumping them into liquid nitrogen. After the boiling ceased the samples were removed with tweezers and transferred quickly into the vacuum lock through the atmosphere. The transfer time into the lock was typically about 1 s, the pump down-time to <1 Pa (10^{-2} mbar) is 10 s. A layer of frost always accumulated on top of the sample during this transfer process, which, however quickly sublimated off the sample for temperatures above 170 K (vide infra). (In the future, more systematic experiments with a dedicated transfer line, which would keep the sample under dry nitrogen throughout, would be desirable.) The frozen samples are then introduced into the main chamber and kept at low temperature. The dried frozen samples form a thin, optically homogeneous and transparent layer, the rim of which is barely visible under microscopic observation (×100). Spectra of superior quality and very good reproducibility can be obtained from all locations within the 3–6 mm diameter sample area; only one spectrum can be recorded from any given location, testifying to the very thin sample layer. Three temperature ranges can be differentiated for the analysis: For temperatures below about 170 K no useful protein signals are observed, presumably because the sample is still covered by frost, which accumulates primarily during the sample introduction into the vacuum. Very good spectra can be obtained in the range of 170–220 K for several hours after sample introduction into the vacuum. For some proteins this temperature range extends as high as 240–270 K. Mass resolution (FWHM) of 300–350 is routinely obtained for proteins up to a molecular mass of 20 kDa in this temperature range; selected spectra exhibit a mass resolution of up to 700 as demonstrated for cytochrome *c* in Fig. 2. This contrasts with a resolution of 150–200 obtainable with the given instrument for standard preparations of the same proteins under optimal conditions in UV- and IR-MALDI. Above these temperatures the sample loses its water of hydration by sublimation or evaporation on a time scale too short for useful mass spectrometry. Good spectra of molecules above 10 kDa typically require a protein concentration of 10^{-3} M; however, spectra have been obtained from 10^{-5} M solutions, although with a substantially decreased signal-to-noise-ratio and a strong low mass signal background. For sample solutions below 10^{-5} M, analysis is also possible if several layers of sample are prepared on top of each other. For pure aqueous solutions, the upper mass limit so far is ≈30 kDa; however, spectra of bovine serum albumin at molecular mass of 66 kDa with a maximum mass resolution of 280 have been obtained from 5×10^{-4} M solutions, buffered with 50 mM Tris·HCl (Fig. 3), which by itself is known to be a functional IR matrix. Salt addition leads to a degradation of signal quality, as is also known for standard preparations, but even a physiological NaCl concentration does not totally suppress the protein signals. Mixture analysis is also possible. Fig. 4 shows a spectrum with insulin (5,734 Da), ubiquitin (8,565 Da), and bovine heart cytochrome *c* (12,360 Da). This can be used for example for internal mass calibration. A mass accuracy of 0.01%, as commonly obtained for these proteins in UV-MALDI-MS, was easily achieved with this procedure. The threshold irradiance (I_0) for protein detection is ≈3×10^6 W/cm^2, quite comparable to values needed for standard preparations in the UV as well as in the IR for preparations with other matrices such as succinic acid. Near I_0, small to sometimes no "matrix" signals are recorded in the low mass range. As commonly observed in MALDI-MS, these matrix signals increase appreciably with increasing irradiance. The same holds true for protein signals up to an

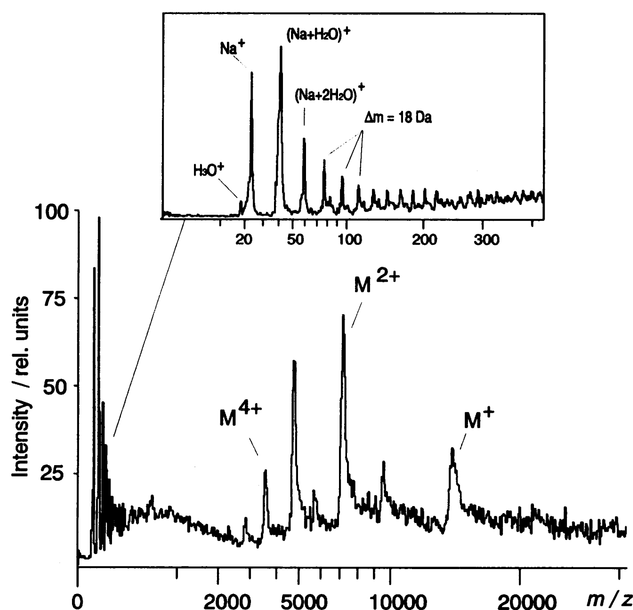

FIG. 1. Mass spectrum of hen egg lysozyme (M_r, 14,305 Da). The sum of 15 single shots. Spectrum obtained from a 10^{-5} M frozen aqueous protein solution.

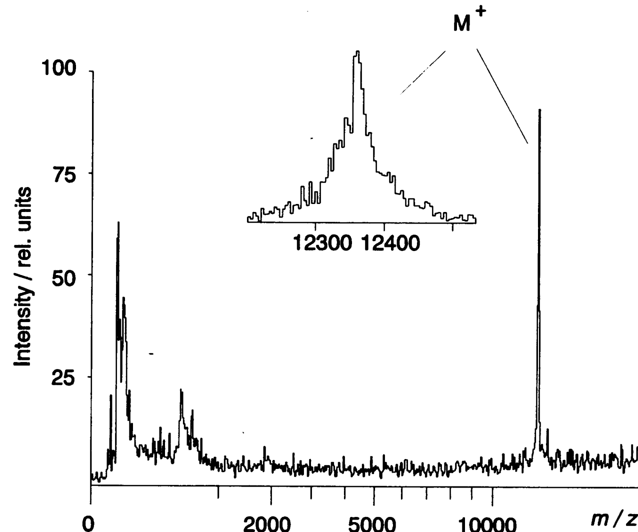

FIG. 2. Mass spectrum of horse heart cytochrome *c* (M_r, 12,360 Da). The sum of 10 shots. Spectrum obtained from a frozen air-dried 10^{-3} M aqueous protein solution.

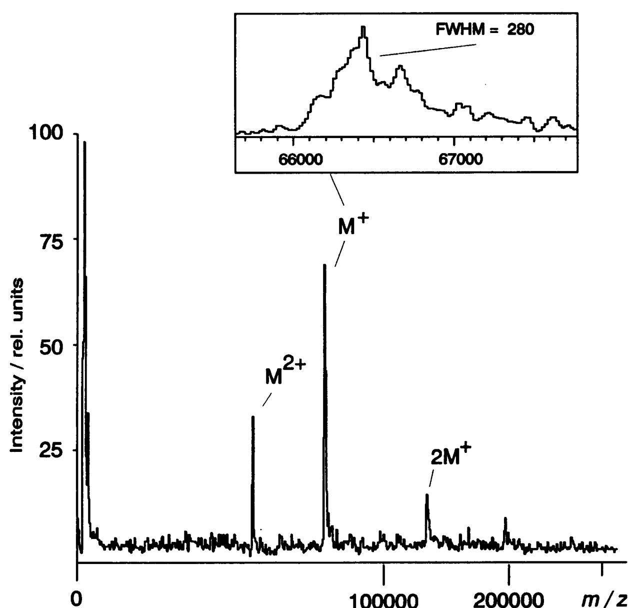

FIG. 3. Mass spectrum of bovine serum albumin (M_r, ≈66 kDa) obtained from a frozen (T = 200 K) air-dried 5×10^{-4} M solution, buffered by 50 mM Tris·HCl. The sum of five single shots.

irradiance of 2–3 I_0 depending somewhat on the specific protein under investigation. Above 2–3 I_0 the protein signal begins to decrease; however, the mass resolution of the protein signal remains essentially unaffected up to about an 8-fold increase in irradiance. This contrasts with results for standard UV- and IR-MALDI analyses, for which the resolution begins to degrade at levels as low as 1.5 I_0 (UV) and 2.5 I_0 (IR).

The exact water content of the frozen samples, i.e., the exact matrix/analyte ratio is not known for the given experimental conditions because the temperature and partial water pressure cannot be independently controlled in the existing apparatus. As a result, the sample cannot be expected to equilibrate with its vacuum environment. The observations, however, suggest that upon freezing to liquid nitrogen temperature samples essentially retain the "bound" water they acquired in the "semidry" or lyophilized state, in equilibrium with the ambient humidity at room temperature. In the temperature range of 170–250 K the diffusion and sublimation rate of the water of hydration is so slow that the majority of this water stays in the sample for hours and good spectra can be obtained.

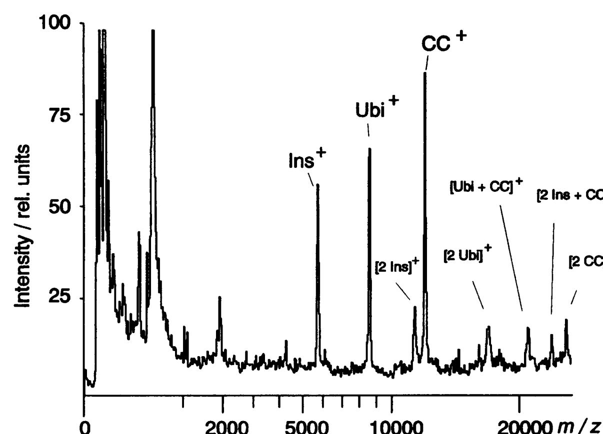

FIG. 4. Mass spectrum of insulin (5,734 Da), ubiquitin (8,565 Da), and bovine heart cytochrome *c* (12,360 Da) obtained from an air-dried, frozen aqueous protein solution (5 μl, 8×10^{-4} M each). The sum of 10 single shots.

The hypothesis that it is essentially the water of hydration that makes a desorption/ionization of the proteins possible was checked by recording spectra directly from protein single crystals. Crystals of lysozyme were grown at 20°C by the hanging drop vapor diffusion method (9, 10). The mother liquor initially contained either 10 g/liter of protein and 10 g/liter of $NaNO_3$ or 20 g/liter of protein and 20 g/liter of PEG 6000, both in 0.01 M aqueous acetate buffer at pH 4.6. The precipitating solution contained either 50 g/liter of $NaNO_3$ or 100 g/liter of PEG 6000 in the same buffer. Crystals of suitable size grew in both solutions over a period of 2–3 months. Crystals from both solutions yielded protein spectra, with the spectra from crystals grown in $NaNO_3$ of somewhat inferior quality (reduced mass resolution and S/N-ratio) due to the salt content as would be expected for standard MALDI analyses. Fig. 5*A* shows a single lysozyme crystal of $\approx 1 \times 1$ mm^2 dimension while still in the mother liquor (PEG 6000). Upon removal of the solution and transfer to the sample holder it fractured into two pieces, before being shock frozen and introduced into the vacuum. Within the temperature range of 170–200 K, spectra were obtained from all accessible locations (all of them on the two top quadrant external faces of the crystal) of fragments (Fig. 6). Fig. 5*B* shows the crystal after warming it back up to room temperature and removing it from the instrument. A number of craters are clearly visible in locations from which up to 20 successive spectra were obtained. The fact that multiple spectra were recorded even from deep inside the crystal is taken as proof that the results cannot have been induced by spurious surface contamination introduced during the crystal transfer into the mass spectrometer.

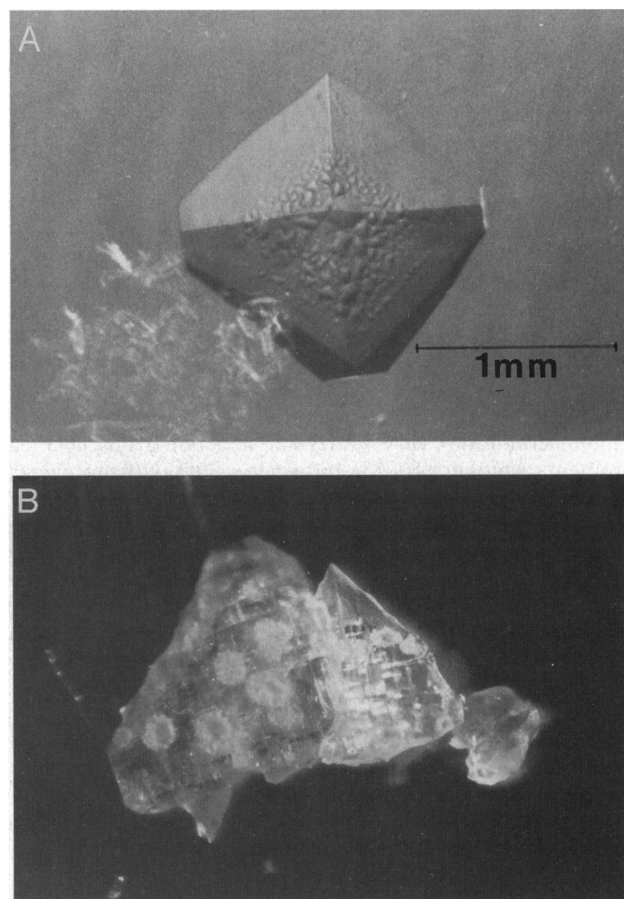

FIG. 5. Lysozyme crystal of ≈1 × 1 mm dimension grown with PEG 6000 as precipitant. Before analysis (*A*) and after analysis (*B*). A number of craters are clearly visible in locations from which up to 20 successive spectra had been obtained.

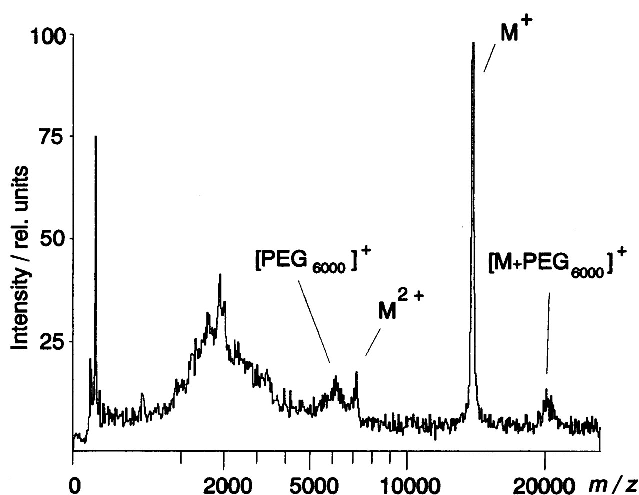

FIG. 6. Mass spectrum of hen egg lysozyme (M_r, 14,305 Da) desorbed from the crystal shown in Fig. 5. The sum of 10 single shots.

DISCUSSION

Besides demonstrating the utility of water as a matrix for IR-MALDI, these results certainly challenge our current understanding of the mechanisms in MALDI ion formation in general, and the role of the matrix in particular. This role is thought to participate in (*i*) acting as the dominant, if not the only, absorber of laser energy, thereby sparing the macromolecules from excessive internal heating; (*ii*) isolating the macromolecules from each other, thereby preventing the formation of large clusters and complexes by protein–protein interaction; and (*iii*) aiding in the ionization of the macromolecules, e.g., by proton transfer. Both this ionization and a substantial cooling of internal degrees of freedom is thought to take place in the adiabatically expanding plume of ablated matrix volume seeded by the macromolecules (11, 12). Matrix isolation and, most probably, cooling in the expanding plume require a substantial molar excess of the matrix versus analyte and, indeed, this ratio has been found to be in the range of typically 10^4-10^5 for standard samples in UV- and IR-MALDI.

The published values for the water content in lysozyme crystals are ≈30% of the total mass (13–15). This corresponds to a molar ratio of water to lysozyme molecules of ≈250, smaller than the above value under standard MALDI conditions by two to three orders of magnitude. In semi-dry lysozyme samples the reported values of hydration under ambient conditions vary somewhat depending on the measurement technique (16–18), but again ≈30% of the total mass appears reasonable. As explained above, this value is an upper limit for the water content of samples, remaining in the vacuum for several hours at temperatures in the range of 170 to ≈250 K because of the slow water loss by sublimation. The molar water/protein-ratio decreases in proportion to the protein mass and this is, most probably, the reason for the observed upper mass limit of 30 kDa for preparations containing only the water of hydration.

Even though proteins in a crystal are still surrounded by solvent, some packing interactions between the protein molecules are certainly present. So, in contrast to standard MALDI conditions, matrix isolation is not fully achieved. It is reported in the literature that at a wavelength near 3 μm the IR absorption of proteins in the crystalline state is dominated by that of water (19), but the abundant O—H and N—H groups of the protein must lead to the deposition of a substantial fraction of the energy into the protein itself. This process is true not only for IR-MALDI in general, but particularly for the relatively low molar ratio of the water matrix to analyte in these crystals. It could be argued that the process is, in reality, driven by the energy deposited into the substrate rather than the sample itself, as suggested by Williams *et al.* (7, 8) for their method. However, while desorbing from the single lysozyme crystal the laser light does not reach the substrate at all and for semi-dried layers of proteins identical results were obtained when the experiments were repeated with a nonabsorbing ZnSe substrate (as was observed earlier for standard preparation in IR-MALDI). Also, comparing copper, aluminum, silver, and stainless steel as an influence of the substrate material was not observed. The laser energy must, therefore, be deposited into the sample itself.

At the same time it is difficult to believe that the dynamics of the expanding plume are largely dominated by less than 30% (vol/vol) water. The fact that mass spectra of proteins have many common features for various desorption techniques including static secondary ion MS (TOF-SIMS) and plasma desorption MS (PD-MS) is usually assumed to result from the extensive chemistry in the expanding plume strongly favoring the most stable reaction products (11). However, predicting what this chemistry should be with protein and water as the only available reactants is quite difficult. Whereas substantial evidence has been accumulated for the active role of the matrix in the ionization step for UV-MALDI (5), where the plume is assumed to contain a sizable concentration of highly reactive (radical) photoionized and electronically excited matrix molecules, an identical role is difficult to imagine for water with its exceptionally high ionization potential of 12.6 eV.

In summary, it would appear that the only common feature of all these techniques is a very rapid energy deposition into the sample. The strong similarity of the resulting mass spectra is either accidental, or the determining mechanistic step in the ion formation processes has not yet been identified. More research is, undoubtedly, necessary to answer these questions.

Although important information on the desorption/ionization process has been gained from these experiments, the practical application of MALDI with an ice matrix currently is still somewhat limited by sensitivity. Femtomole and even subfemtomole amounts of protein have been used successfully for UV-MALDI preparations and attomole amounts are typically consumed per single spectrum (20, 21). In IR-MALDI, the amounts of protein needed for preparation and consumed per single spectrum are typically a factor of 100 higher. With a minimum volume for the preparation of 0.1 μl, 100 pmol of protein would be needed; the amount desorbed per laser shot is estimated to be around 1 pmol. At the present state, a minimum amount of ≈10 pmol is needed for a successful protein analysis. This fact, together with the recent observation that below a spot size of ≈100 μm MALDI mass spectrometry becomes increasingly difficult (22), certainly casts some doubt on the feasibility of microprobing the subcellular distribution of macromolecules in biological specimens. This remains a challenge for future research which must first improve the detection limit by a suitable variation of the experimental parameters.

CONCLUSION

We have demonstrated that IR-MALDI from lyophilized protein, air-dried protein solutions, or protein crystals up to a molecular weight of 30 kDa is possible. Investigations have shown that samples must be frozen to retain a sufficient fraction of the water of hydration that plays a crucial role in the process. However, in particular, the extremely low molar matrix/analyte ratio distinguishes water from other known matrices, commonly used in UV- as well as IR-MALDI. All mechanistic and chemical functions during desorption—and ionization—that are usually attributed to the matrix do not seem to apply for water, or at least not in the same way. Also, water is naturally associated with almost all proteins, which differentiates it from all other matrices. Low sensitivity at the

moment seems to limit the general applicability of water as a matrix in IR-MALDI.

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