

Influence of Matrix Solution Conditions on the MALDI-MS Analysis of Peptides and Proteins

Steven L. Cohen and Brian T. Chait*

Laboratory for Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10021

Sample–matrix preparation procedures are shown to greatly influence the quality of the matrix-assisted laser desorption/ionization (MALDI) mass spectra of peptides and proteins. In particular, dramatic mass discrimination effects are observed when the matrix 4-hydroxy- α -cyanocinnamic acid is used for analyzing complex mixtures of peptides and proteins. The discrimination effects are found to be strongly dependent on the sample–matrix solution composition, pH, and the rates at which the sample–matrix cocrystals are grown. These findings demonstrate the need to exercise great care in performing and interpreting the MALDI analysis of biological samples. The results also indicate that there is a reverse-phase chromatographic-like dimension in the sample–matrix preparation procedures that can be exploited to optimize the analysis. The present work describes the conditions under which the majority of components of a complex mixture of peptides and proteins can be successfully measured.

It is generally accepted that factors such as the matrix solvent composition and sample–matrix preparation procedures greatly influence the quality of the matrix-assisted laser desorption/ionization (MALDI) mass spectra of biopolymers. To date, a number of different sample–matrix preparation methods have been developed. The earliest and most widely used procedure for sample–matrix preparation is the dried-drop method.¹ Subsequently, significant improvements in the tolerance of MALDI to high levels of involatile additives were demonstrated using a slow matrix crystallization technique.² More recent studies have demonstrated the uses of a polycrystalline thin film of matrix as a useful preparation procedure.^{3,4} In addition, other factors such as the choice of matrix^{5–7} and matrix additives^{7,8} have been shown to significantly influence the MALDI-MS response of peptides and proteins. An understanding, however, of the influences of other factors such as the matrix solvent composition on the quality of the MALDI spectra have been lacking. To this end, we undertook a detailed investigation of 4-hydroxy- α -cyanocinnamic acid

(4HCCA),⁹ a widely used matrix for the MALDI mass spectrometric analysis of peptides and proteins. We observe dramatic mass discrimination effects in the MALDI-MS response of peptides, proteins, and their mixtures and demonstrate that the discrimination effects are greatly influenced by the composition of the sample–matrix solvent and by the time scales used for growing the sample–matrix cocrystals. These findings allow us to optimize sample–matrix preparation procedures, permitting a successful analysis of the majority of components in a complex mixture of peptides and proteins.

EXPERIMENTAL SECTION

Materials. Matrix Solutions. The matrix used in all experiments was 4-hydroxy- α -cyanocinnamic acid,⁹ obtained from Aldrich (Milwaukee, WI). The water used for the matrix and sample solution preparations was obtained from a MilliQ UV Plus water purification system from Millipore (Bedford, MA). Acids and organic solvents were HPLC grade or better. Sequencing grade endoprotease GluC (*Staphylococcus aureus* V8) was purchased from Promega (Madison, WI). *N*-Octylglucoside was from Boehringer Mannheim (Indianapolis, IN).

The Max Protein. The Max protein used in the experiments consisted of a construct that spans the basic–helix–loop–helix–leucine zipper portion (Max 22–113; 92 residues)¹⁰ of the full-length protein (160 residues).^{11,12} The truncated, recombinant form of Max was expressed in *Escherichia coli* and purified as described elsewhere.¹⁰ This form of the protein (MW = 10 826) has the following sequence: ADKRAHNALE³RKRRDHKIDS-FHSLRDSVPSLQGE⁵KASRAQILDKATE⁴YIQYMRRKNHT-HQQDIDDLKRQNALLE⁶QQVRALE¹KARSSAQLQT². The five glutamate residues (shown underlined in the sequence above) are the principal targets of V8 proteolysis. Superscript numbers, placed in the sequence adjacent to the Glu residues, identify the C-terminals of the six peptide fragments (peaks 1–6 in Figure 3) that would arise from a complete V8 digest of the protein.

Other Proteins. Cytochrome *c* (bovine) was purchased from Sigma (St. Louis, MO). Recombinantly expressed p42/p62, human Ob1, TATA binding protein, and TFIIB protein were kindly provided by Dr. Stephen K. Burley.

Proteolysis of Max. A complete description of the proteolysis protocols is given elsewhere.¹³ Briefly, a 30 μ M sample of Max

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in 50 mM ammonium phosphate buffer (pH 6) was subjected to V8 proteolysis at 25 °C using a V8:Max ratio of 1:20 (w/w). Aliquots (0.5 μ L) of the digest solution were mixed with 15 μ L of MALDI matrix solution (see below) after 30 s (for partial proteolysis) or 1 h (for complete proteolysis) of digestion. The acidity of the matrix solution (pH <3) was sufficient to completely quench any further digestion.

Matrix Solution Preparation. All of the matrix solutions were saturated with 4HCCA and were prepared by adding 4HCCA (solid) to the organic solvent, followed by the addition of water and acid (as required). Each mixture was thoroughly vortexed and centrifuged, leaving a clear working matrix solution. The solubilities of 4HCCA were dependent on the solvent composition, ranging from a low of 5 mM (water/methanol, 2:1 v/v), to an intermediate value of 29 mM (formic acid/water/2-propanol, 1:3:2 v/v/v), to a high value of 74 mM (water/acetonitrile, 1:1 v/v). We note that matrix solutions which contained formic acid (at levels \leq 17% by volume) did not cause any peptide formylation side reactions. *N*-Octylglucoside (critical micelle concentration, 23 mM¹⁴) was added to the matrix solution as necessary. All pH measurements of the matrix solutions were performed at room temperature with a combination pH microelectrode (Model MI-415; Microelectrodes, Inc., Bedford, NH) and pH meter (Model PHM 95; Radiometer, Copenhagen, Denmark).

Sample–Matrix Crystallization Procedures. To prepare the sample–matrix solution, an aliquot (0.5 μ L) of protein or protein digest (30 μ M) was combined with 15 μ L of matrix solution in a small microcentrifuge tube (PGC Scientifics, Gaithersburg, MD), giving a final concentration of 1 μ M per component. Sample–matrix cocrystals were obtained by one of the following techniques.

Dried-Drop Method.¹ An aliquot (0.5–1 μ L) of the sample–matrix solution was deposited onto an aluminum 10-sample MALDI probe tip and allowed to air-dry (several minutes) at room temperatures, resulting in a uniform layer of fine granular matrix crystals. Cold water was placed over the crystals for 10 s to help remove involatile salts. The water was subsequently removed with vacuum suction.

Slow Crystallization Method.² Approximately 15–30 μ L of the sample–matrix solution was left at room temperature in a closed microcentrifuge tube. A small hole (\sim 1 mm diameter) was punched through the cap of the tube, allowing for a partial evaporation of the matrix solution at room temperature. After 8–12 h, a portion of the solution has evaporated, leaving small granular crystals of matrix along the sides of the tube in the remaining supernatant solution. The supernatant was removed, leaving the crystals on the walls of the tube. Cold water was added to the tube to wash the crystals, a step that was repeated 2–3 times. The crystals were scraped off the walls of the tube with a small pipet tip and taken up in \sim 1 μ L of water. The resulting suspension of crystals was deposited directly onto the MALDI probe tip.

Rapid Crystallization Method.¹⁵ An aliquot (0.5 μ L) of the sample–matrix solution was deposited onto the MALDI probe tip, which was immediately put into a vacuum (\sim 20 mTorr) that was maintained by a mechanical rotary pump. The matrix solution evaporated within 12 s, leaving a fine powdery layer of matrix

crystals. With evaporation times much beyond 20 s, the rapid crystallization effects (discussed in the paper) become less pronounced. Peptides and proteins analyzed with the rapid crystallization method tend to exhibit extensive alkali cation adduction. The adduction was substantially reduced by rinsing the crystals on the probe with several aliquots of cold water (see above).

Mass Spectrometry. MALDI-MS was obtained on a linear time-of-flight instrument, a full description of which can be found elsewhere.^{16–18} Briefly, the multiple-sample probe tip was inserted into the time-of-flight instrument ion source and allowed to reach high vacuum. Background pressure within the instrument, measured by an ion gauge located below the source, was better than 3×10^{-7} Torr. A Nd–YAG laser (Lumonics Inc., Ontario, Canada) was set to deliver 355 nm wavelength pulses (\sim 10 ns duration) onto the sample at a rate of 2.5 Hz. Each laser shot produced a full mass spectrum. The spectra presented in this paper represent averages of 100–200 laser shots. The ion acceleration potential was +30 kV, and the flight tube length was 2 m. Ion detection and signal amplification were through a conversion microchannel plate detector–discrete dynode multiplier assembly.¹⁹ The amplified signal was visually monitored with a digital oscilloscope (Model 7200A, LeCroy Corp., Chestnut Ridge, NY), digitized by a transient recorder (LeCroy, Model TR8828D), and stored as time-of-flight data on a Vax 4000 workstation (Digital Equipment Corp., Woburn, MA). The time-of-flight data were either externally calibrated or mass converted using ion peaks of known masses.

RESULTS

Effects of the Matrix Solution Composition. We have found striking effects of the matrix solution composition on the MALDI-MS of peptide and protein mixtures. An example of these effects is illustrated in Figure 1, which compares mass spectra of a mixture consisting of peptides and proteins prepared in three different 4HCCA matrix solutions using the dried-drop method¹ (see Experimental Section). The mixture of peptides was prepared by partial V8 protease digestion of Max,^{11,12} a 10.8 kDa protooncogenic transcription factor (see Experimental Section and ref 13). Figure 1a shows the spectrum of the digest obtained from a matrix solution consisting of a 1:3:2 (v/v/v) mixture of formic acid/water/2-propanol (FWI) (pH 1.3) saturated with 4HCCA, a formulation that has been used with considerable success in our laboratory for protein analysis.^{20,21} Figure 1b shows the results obtained from a saturated 4HCCA solution made with a 2:1 (v/v) mixture of 0.1% trifluoroacetic acid/acetonitrile (TFA/ACN) with pH 2.0.¹⁶ The TFA/ACN matrix solution formulation is commonly used for peptide and protein analysis by many laboratories. A comparison of parts a and b Figure 1 shows that the MALDI

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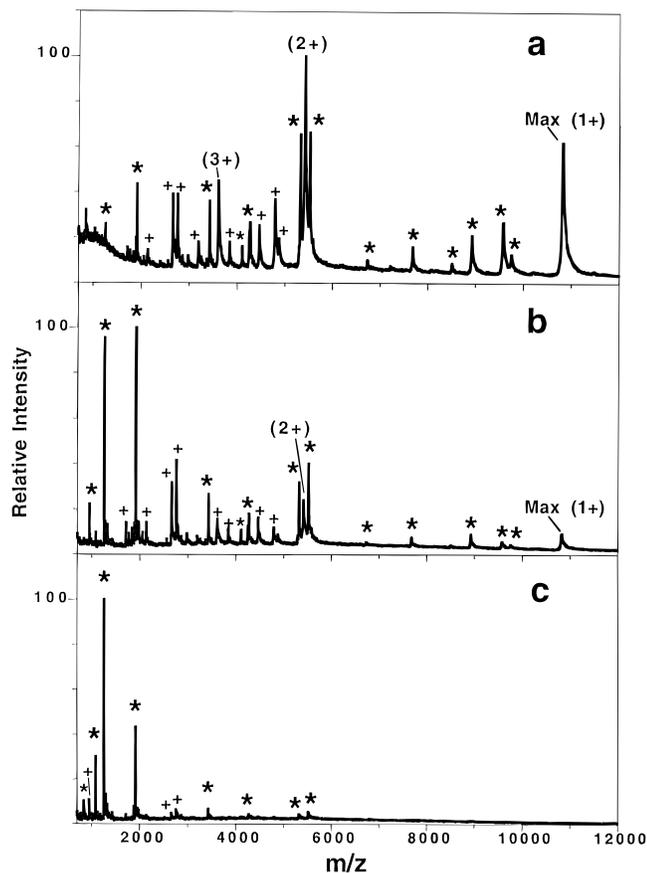


Figure 1. Comparison of the positive ion MALDI mass spectra of the partial V8 digest of Max obtained from three different 4HCCA matrix solutions using the dried-drop method. The matrix solution compositions consist of (a) formic acid/water/2-propanol (1:3:2 v/v); (b) 0.1% trifluoroacetic acid/acetonitrile (2:1 v/v); and (c) water/acetonitrile (2:1 v/v). Both partial and complete digest fragments are observed. Peaks that are marked 1+, 2+, and 3+ designate singly, doubly, and triply protonated undigested Max. Peaks arising from singly protonated fragments are marked by asterisks; multiply protonated fragments are denoted by crosses. A full description of the fragments can be found in ref 13.

response from the sample prepared in the FWI solution favors the appearance of high-mass components (>6 kDa) compared to the response from that prepared in the TFA/ACN solution. The third spectrum (Figure 1c) was obtained from a saturated 4HCCA solution consisting of a 2:1 (v/v) mixture of water/acetonitrile (pH 2.5), where the acidity derives from the moderately acidic matrix. This latter preparation yields a spectrum dominated by low-mass peaks (<2 kDa) and devoid of high-mass peaks (>6 kDa). We have analyzed partial digests of other proteins (e.g., cytochrome *c*, the human Ob1 protein²²) using the three 4HCCA matrix preparations described above and have found mass discrimination effects similar to those presented in Figure 1 (data not shown).

The differences in the MALDI response in Figure 1 can be attributed to factors relating to the matrix solution composition and pH. These effects were further explored by examining the mass spectrometric response of the Max digest using a large variety of matrix solution compositions (Table 1). The acidities of these matrix solutions range from pH 1.1 (4HCCA–0.1 N HCl/

Table 1. Effects of Matrix Solution Composition and pH on the MALDI-MS of a Complex Mixture of Peptides^a

compos of 4HCCA solution	pH	response ^c
0.1 N HCl/ACN (2:1) ^b	1.1	(l, i, h)
1% TFA/ACN (2:1)	1.1	(l, i, h)
formic acid/water/ACN (1:3:2)	1.1	(l, i, h)
formic acid/water/IPA (1:3:2)	1.3	(l, i, h)
0.5% TFA/ACN (2:1)	1.4	(l, i, h)
formic acid/water/MeOH (1:3:2)	1.6	(l, i, h)
0.01 N HCl/ACN (2:1)	1.9	l, i, h
formic acid/water/IPA (0.2:3:2)	2.0	l, i, h
0.1% TFA/ACN (2:1)	2.0	l, i, h
0.1% TFA/MeOH (2:1)	2.0	l, i, h
0.1% TFA/IPA (2:1)	2.1	l, i, h
2 M acetic acid/ACN (2:1)	2.3	l, i, h
water/ACN (1:1)	2.4 ^d	l, i, (h)
water/ACN (2:1)	2.5 ^d	l, i, (h)
water/IPA (2:1)	2.7 ^d	l, i, (h)
water/EtOH (2:1)	2.8 ^d	l, i, (h)
water/MeOH (2:1)	2.9 ^d	l, i, (h)

^a Peptide mixture is a partial V8 protease digest of the 11 kDa protein Max. The sample–matrix preparation procedure used is the dried-drop method. ^b Volume ratio. ^c Response refers to a measure of relative peptide intensity, estimated from the mass spectra obtained for each matrix solution. The response is divided over three mass ranges designated by the letters l (low, 0.8–2 kDa), i (intermediate, 2–6 kDa), and h (high, 6–11 kDa). For each solution, the three mass ranges (l, i, or h) are assigned a response level: letters in boldface designate the mass range exhibiting the strongest relative response; letters in normal type indicate a moderate response; and letters in parentheses indicate a weak or no response. ^d The acidity of these solutions derives from the moderately acidic 4HCCA.

acetonitrile) to pH 2.9 (4HCCA–water/methanol). The results listed in Table 1 follow the same trends observed in Figure 1, showing a strong correlation between the matrix solution acidity and the MALDI-MS response to the different components in the digest. Highly acidic matrix solutions (pH <1.8), regardless of the identity of the added acid or organic solvent, showed weak or no signal for peptides <2 kDa and mainly favored the appearance of components with masses >2 kDa. In addition, matrix solutions that contained formic acid and had pH <1.8 consistently yielded the strongest response to high-mass components. Matrix solutions with pH between 1.8 and 2.3 exhibited mass spectrometric peaks that spanned the greatest latitude in mass range, although tending to favor the low- and intermediate-mass over the high-mass components. The matrix solutions with pH >2.3 (i.e., no added acids), regardless of the identity of the organic solvent, consistently showed few components above 6 kDa and strongly favored the appearance of peptides below 2 kDa.

Effects of the Detergent *N*-Octylglucoside. We speculated that the above-described pH-dependent discrimination effects (Figure 1 and Table 1) arise from differences in polypeptide solubility and tested this hypothesis by monitoring the effects of the addition of *N*-octylglucoside on the MALDI-MS response of the V8 digest of Max. *N*-Octylglucoside is a nonionic detergent that is known to aid in protein solubilization.²³ It has been previously shown that the presence of *N*-octylglucoside in the matrix solution enhances the MALDI-MS response of the larger peptides in digest mixtures.²⁴ Figure 2 displays the MALDI spectra of the V8 digest of the Max protein run in the absence (Figure 2a) and presence (Figure 2b) of 20 mM *N*-octylglucoside

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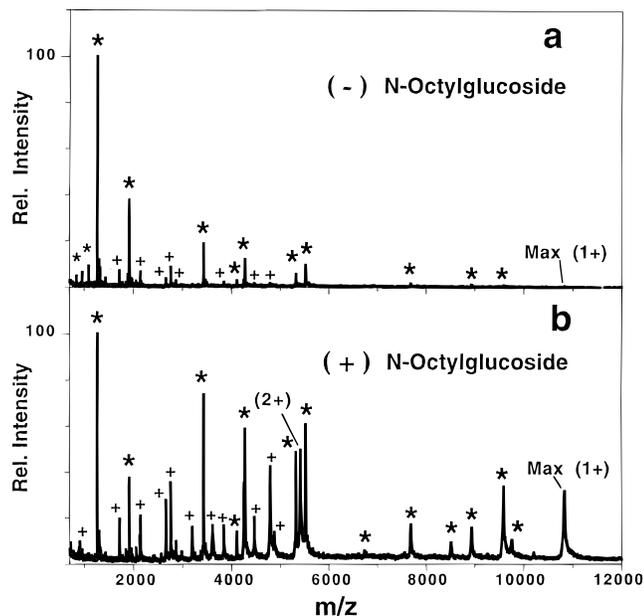


Figure 2. Effects of *N*-octylglucoside on the MALDI mass spectra of the partial V8 digest of Max. Spectra were obtained from a matrix solution consisting of 4HCCA–water/acetonitrile (2:1 v/v) in the (a) absence or (b) presence of 20 mM *N*-octylglucoside using the dried-drop method. See Figure 1 caption for a description of the symbols.

in a 4HCCA–water/acetonitrile (2:1 v/v) matrix solution. In the presence of *N*-octylglucoside, the high-mass discrimination effects are significantly reduced. Virtually all of the high-mass polypeptides are “restored” in the spectrum (Figure 2b), which now more closely resembles the spectrum obtained with the FWI matrix solution (Figure 1a). These results suggest that the observed pH-dependent matrix solution mass discrimination effects may be related to polypeptide solubility.

Effects of the Rate of Matrix Crystal Growth. In addition to the matrix solvent composition, an important element of the sample–matrix preparation procedure is the matrix crystallization step. The most commonly used strategy to grow analyte-doped matrix crystals involves variations of the dried-drop method, a procedure that usually depends on a moderately quick (minutes) and complete evaporation of a small drop of matrix solution.¹ A second approach in growing matrix crystals employs a method involving a much slower (hours) matrix crystallization,² in which the matrix solution is allowed to partially evaporate, a process that is accompanied by matrix crystal formation within the remaining matrix solution. The slow crystallization technique is used to overcome suppressive effects on the matrix crystallization process arising from high levels of involatile additives that are frequently found in biological samples.² In addition to the dried-drop and slow crystallization procedures, a third approach, involving a rapid (seconds) evaporation of a small drop of matrix solution, can be used to obtain matrix crystals.¹⁵

Using the three matrix preparation methods, we have explored the quality of the MALDI-MS response when utilizing different time scales to grow sample–matrix crystals. An example of our findings is given in Figure 3, which compares MALDI spectra of a peptide mixture using the slow, dried-drop, and rapid crystallization methods for two different matrix solution compositions. The peptide mixture derives from a *complete* V8 digest of Max. (The complete digest of Max produces principally six peptide fragments, labeled 1–6 in Figure 3; also see Experimental

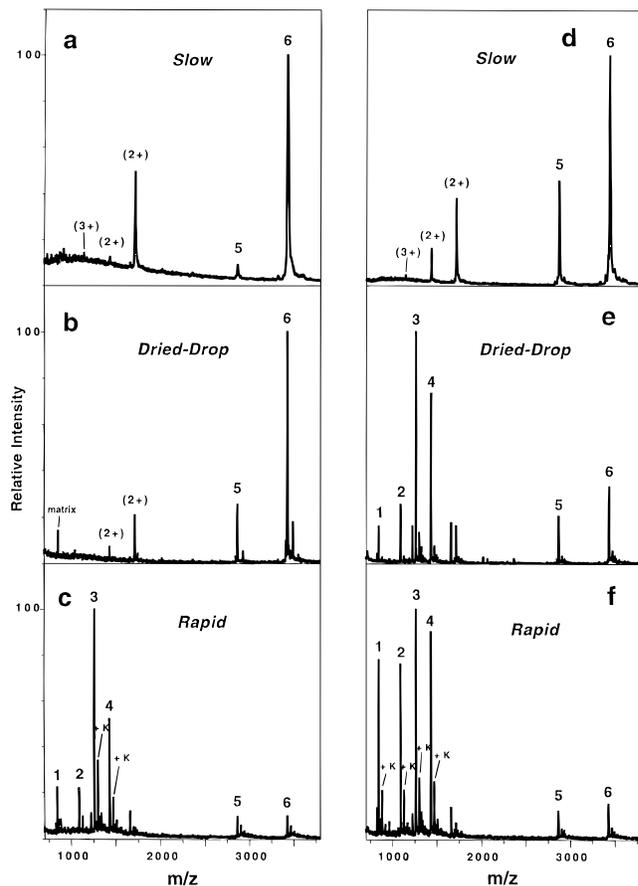


Figure 3. Effects of the rate of matrix crystal growth. Positive ion spectra of the complete V8 digest of Max obtained from two matrix solutions: (a–c) 4HCCA–formic acid/water/2-propanol (1:3:2 v/v/v) and (d–f) 4HCCA–water/acetonitrile (2:1 v/v). Results using three rates of matrix crystallization are illustrated: top panels (a,d), slow crystallization; middle panels (b,e), dried-drop; and bottom panels (c,f), rapid crystallization. A complete V8 digestion of Max results in six fragments that appear in the spectra as singly protonated peptides (labeled 1–6). Multiply protonated fragments are indicated as 2+ and 3+.

Section.) The mass spectra in Figure 3a–c were obtained from a sample–matrix solution made from the 4HCCA–formic acid/water/2-propanol (1:3:2 v/v/v) formulation, while spectra in Figures 3d–f were obtained from a sample–matrix solution made from 4HCCA–water/acetonitrile (2:1 v/v). The spectrum in Figure 3a, obtained from the FWI matrix solution using slow crystallization, shows exclusively high-mass peptide fragments 5 and 6 and is dominated by the latter. No traces of fragments 1–4 appear in the spectrum. The spectrum in Figure 3b, obtained from the same FWI solution using the dried-drop method, also shows only fragments 5 and 6. In stark contrast, the spectrum in Figure 3c, obtained from the FWI solution using the rapid crystallization technique, shows all six fragments, a strong indication that rapid matrix crystallization can “overcome” the low-mass discrimination effects observed from either slow crystallization (Figure 3a) or the dried-drop methods (Figure 3b).

Results using the 4HCCA–water/acetonitrile matrix solution (Figure 3d–f) reveal characteristics similar to those observed with FWI. The spectrum in Figure 3d, obtained using slow crystallization, shows only the two high-mass fragments 5 and 6 (cf Figure 3a), whereas the spectrum in Figure 3e, obtained from the dried-drop method, shows all six fragments. The spectrum

in Figure 3f, obtained from rapid crystallization, exhibits all six fragments and is dominated by the lower mass fragments 1–4.

The results illustrated in Figure 3 indicate that the rates of matrix crystal growth have striking effects on the MALDI-MS response of different mass components in a polypeptide mixture. We have performed similar experiments with other polypeptides, including a synthetic peptide ladder and enzymatic digestions of the human Ob1 protein²² (data not shown). The results from these experiments mirror the findings illustrated in Figure 3 and are summarized as follows. (1) The slow matrix crystallization technique favors the observation of high-mass components over the low-mass peptides, regardless of the composition and pH of the matrix solution (Figure 3a,d). (2) Using the dried-drop method of crystallization, a strong pH-dependent mass discrimination effect is observed, as illustrated in Figures 1, 2, and 3b,e and Table 1 and as described in the previous sections. (3) Rather strikingly, the use of the rapid crystallization procedure favors the observation of low-mass peptides, regardless of the matrix solution composition and pH.

Mass Discrimination Effects between Proteins. We have shown that there are appreciable MALDI-MS mass discrimination effects from complex mixtures of peptides and proteins (Figures 1–3). The components of these mixtures ranged in mass from 0.8 to 11 kDa. We have also observed strong mass discrimination effects between small proteins whose masses differ from each other by <1 kD. An example of this phenomenon is illustrated in Figure 4, which compares spectra run under various conditions of a protein that consists of two noncovalently interacting subunits with molecular masses 9.0 and 9.9 kDa. These subunits are recombinant, truncated forms of the proteins p62 and p42, factors that have been implicated in activated transcription.²⁵ The proteins share similar amino acid composition: the number of positively charged residues in p42 and p62 are 14 and 14, respectively; negatively charged residues, 13 and 11; polar uncharged residues, 21 and 22; and nonpolar residues, 38 and 36. The *pI* (isoelectric point) values were also estimated:²⁶ p42, *pI* = 6.5, and p62, *pI* = 9.9.

Figure 4 compares the MALDI response of the proteins with use of two matrix crystallization methods run in three different matrix solution preparations. Figure 4a–c shows results of the dried-drop method, while Figure 4d–f shows results using the slow crystallization procedure. The three preparations consist of 4HCCA in formic acid/water/2-propanol (1:3:2 v/v/v; FWI) (Figure 4a,d); 0.1% TFA/ACN (2:1 v/v) (Figure 4b,e); and 0.1% TFA/ACN with 8 mM *N*-octylglucoside (Figure 4c,f). The spectra reveal dramatic discrimination effects that are influenced by the matrix solution composition and the rates of growth of the matrix crystals. The spectrum obtained from the FWI matrix solution and the dried-drop method (Figure 4a) shows results that approach the 1:1 stoichiometry expected for the p42 and p62 subunits of the protein. In contrast, the spectrum obtained from the TFA/ACN matrix solution using the dried-drop method (Figure 4b) shows a strong signal from p62 and a weak signal for p42. The addition of *N*-octylglucoside to the TFA/ACN matrix solution restores the response of p42 (Figure 4c) to levels similar to that observed with FWI (Figure 4a). We contrast the dried-drop results (Figure 4a–c) with our findings using the slow

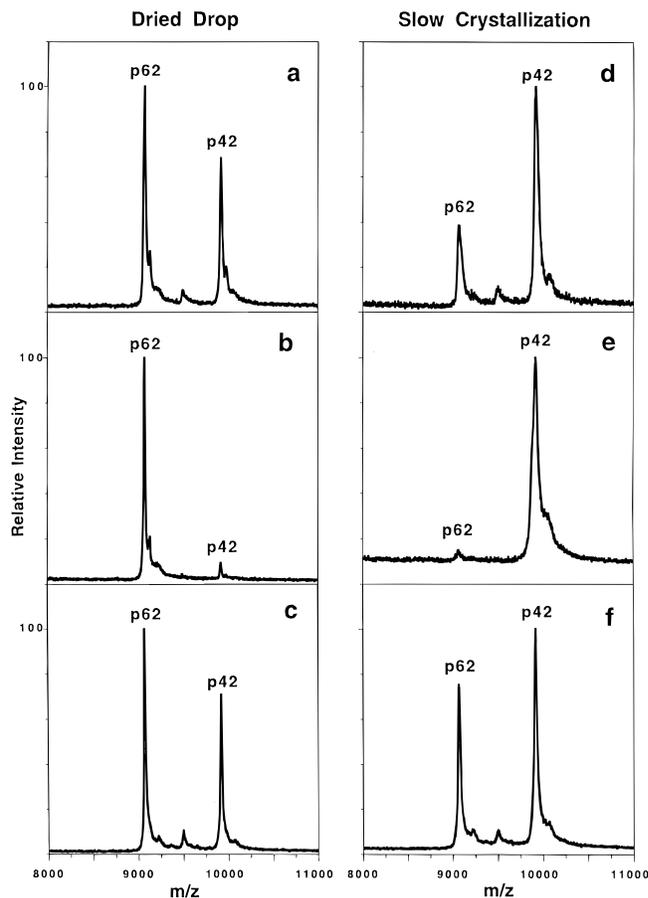


Figure 4. Mass discrimination effects between truncated forms of the proteins p42 and p62. MALDI mass spectra were obtained using the dried-drop method (left column) or the slow crystallization method (right column). Spectra were obtained from three 4HCCA matrix solutions: top panels (a,d), formic acid/water/2-propanol (1:3:2 v/v/v); middle panels (b,e), 0.1% trifluoroacetic acid/acetonitrile (2:1 v/v); and bottom panels (c,f), 0.1% trifluoroacetic acid/acetonitrile (2:1 v/v) with 8 mM *N*-octylglucoside. Only a portion of the spectra that display the singly protonated proteins is shown.

crystallization method (Figure 4d–f). Again, discrimination effects are observed between the two proteins but, surprisingly, in reversed order compared to the dried-drop method (compare Figure 4a with 4d and 4b with 4e). The addition of *N*-octylglucoside in the TFA matrix solution again restores the response of the discriminated subunit—this time p62 (Figure 4f).

We observe discrimination effects using other 4HCCA matrix solution compositions (such as those listed in Table 1) between p42 and p62 as well as between other proteins (e.g., Max, TATA binding protein, and TFIIB) (data not shown). In general, for mixtures of proteins of comparable masses that exhibit a propensity toward the discrimination effects illustrated in Figure 4, we find that (1) matrix solutions that are prepared with formic acid and have pH <1.8 are particularly effective in yielding MALDI spectra that more closely reflect the true stoichiometries of the proteins in solution; (2) matrix solutions containing TFA, acetic acid, or HCl exhibit a tendency toward mass discrimination between the proteins; and (3) a moderate level of *N*-octylglucoside in the matrix solution can often reduce the mass discrimination effects.

DISCUSSION

We have demonstrated that MALDI-MS analysis of complex mixtures of polypeptides (using the matrix 4HCCA) is ac-

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accompanied by strong mass discrimination effects that are related to the sample–matrix preparation procedures. These effects are highly dependent on the rates at which the sample–matrix cocrystals are grown—slow, rapid, or intermediate (dried-drop).

Slow (Hours). The slow crystallization procedure favors the appearance of high-mass components of complex mixtures, regardless of the matrix solution composition and pH (see Figure 3a,d). We hypothesize that during matrix crystallization, polypeptide components partition between the growing matrix crystal lattice and the bulk solution. The tendency to partition between the crystal and the solution suggests a resemblance to the partitioning of polypeptides between a reverse-phase column and the solution phase in reverse-phase liquid chromatography. This idea is based upon a detailed physicochemical investigation of the matrix sinapinic acid (a cinnamic acid derivative) undertaken by Beavis and Bridson.²⁷ In this study, the authors showed that polypeptides were attaching to the (10 $\bar{3}$) face of the growing matrix crystal, a surface that was determined to be nonpolar. They thus believed that “hydrophobic” interactions could be a significant mode of polypeptide attachment to the matrix crystal. Borrowing from the concepts of reverse-phase liquid chromatography, the propensity of a polypeptide to adhere to the matrix crystal lattice would be an inverse function of the mass of the peptide, a relationship that is consistent with our findings that show crystallization strongly discriminates against the incorporation of low-mass components of mixtures.

Rapid (Seconds). The rapid crystallization method favors the appearance of low-mass peptides, regardless of the composition or pH of the matrix solution (Figure 3c,f). We believe that under rapid crystallization conditions, there is insufficient time for extensive partitioning of peptide components between the growing matrix crystal and the solution phase (as postulated for slow crystallization). Under these circumstances, both low- and high-mass components in solution quickly become “captured” in the matrix crystals, a phenomenon that would be largely independent of the matrix solution composition and pH. Spectra obtained from rapid crystallization of complex mixtures of peptides show a relatively stronger response for low-mass than for high-mass peptide components. The difference in response between high- and low-mass peptides probably reflects a greater MALDI-MS sensitivity for the low-mass peptides.

Dried-Drop (Minutes). Unlike the findings observed from the slow and rapid crystallization methods, the dried-drop method exhibits strong pH- and composition-dependent mass discrimination effects (Figures 1, 2, and 3b,e and Table 1). We cannot fully rationalize these results. Although the microscopic events within a small drying drop of matrix solution have not been well characterized, a number of phenomena are likely to be important: (1) The organic solvent is expected to volatilize quickly from a drying drop of matrix solution, leaving the solution enriched in the aqueous phase. (2) The pH of the droplet changes during its evaporation (S. L. Cohen, unpublished results). These results indicate that the pH of a small drop of matrix solution, prepared with added acid solvents, decreases with droplet evaporation, whereas the pH of a drop of matrix solution, prepared without added acid, increases during drop evaporation. (3) Related to (1) and (2) above are polypeptide solubility and a partitioning between the solution and matrix crystal phases. The results using *N*-octylglucoside suggest that polypeptide solubility plays an

important role in the discrimination effects, especially from matrix solutions with pH >2.3 (Figure 2). In addition, peptide solubility may be the reason for the particular benefits of using formic acid in the matrix solution, since formic acid is known for its strong polypeptide solvating properties. (4) It is not clear why highly acidic matrix solutions (pH <1.8), regardless of the identity of the acid, would cause discrimination against the low-mass peptides (compare parts b and e of Figure 3). The low-mass discrimination may reflect a partitioning phenomenon as described for slow crystallization. In this scheme, low-mass peptides would favor remaining in the acidic matrix solution instead of partitioning into the growing matrix lattice.

There are other factors that influence the MALDI-MS response, including peptide polarity and basicity.²⁸ Highly acidic peptides often yield poor or no response by MALDI-MS (S. L. Cohen, unpublished results, and ref 29). The matrix solution discrimination effects that we have characterized are observed irrespective of these other factors.

We include a note pertaining to the interpretation of our findings as concerns quantitation and relative versus absolute intensity of mass spectral peaks. First, our data interpretation has been based on the premise that the matrix preparation procedures affect mainly the extent to which an analyte incorporates into the matrix and on the assumption that such incorporation does not significantly affect the laser desorption and ionization properties of the analyte. Second, accurate quantitation of peptides and proteins by MALDI-MS is often quite difficult, highly dependent on the laser fluence levels and the nature of the polypeptide (unpublished results and ref 30). In all of our experiments, the laser fluence was set slightly above the desorption/ionization threshold and varied only marginally between samples. We have reduced the dependence on quantitation and on the need for absolute sensitivity measurements by relying on comparative analyses of the mass spectra of samples consisting of the same components and interpreting the data on the basis of changes in the relative peak heights. Any small variation in laser fluence did not greatly affect the relative intensities.

CONCLUSIONS

A major strength of MALDI mass spectrometry is its facility for analyzing complex mixtures of peptides and proteins. We have demonstrated that the choice of the matrix solvent system and the rate of matrix crystal growth add a certain chromatographic-like dimension to the sample–matrix preparation and that to take full advantage of the power of MALDI, careful attention must be paid to the sample–matrix preparation procedures. Having extensively investigated these effects, we suggest here some general guidelines for matrix solution preparation (using the matrix 4HCCA and the dried-drop method): (1) The analysis of proteins and high-mass peptides (>3 kDa) is best performed using matrix solutions that include formic acid and have a pH <1.8. (2) The analysis of small peptides (<3 kDa) is best carried out using matrix solutions that do not have added acids. (3) For a complex mixture of peptides and proteins, a single matrix solution (pH ~2) can be used (such as TFA/ACN). However, to obtain a more complete coverage of components in the mixture, it is advisable to run the mixture separately in the two matrix solutions

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suggested in (1) and (2) above. Since the optimization may be sample dependent, we recommend a fine tuning of the matrix solvent composition.

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