4-Chloro- α -cyanocinnamic acid is an advanced, rationally designed MALDI matrix

Thorsten W. Jaskolla^{†‡}, Wolf-Dieter Lehmann[§], and Michael Karas^{†‡¶}

[†]Cluster of Excellence Macromolecular Complexes, Institute for Pharmaceutical Chemistry, Johann Wolfgang Goethe-University, Max-von-Laue-Strasse 9, 60438 Frankfurt, Germany; and [§]German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

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Matrix-assisted laser desorption/ionization (MALDI) has become an enabling technology for the fields of protein mass spectrometry (MS) and proteomics. Despite its widespread use, for example, in protein identification via peptide mass fingerprinting, a comprehensive model for the generation of free gas-phase ions has not yet been developed. All matrices in use today, such as α -cyano-4-hydroxycinnamic acid (CHCA), have been found empirically and stem from the early days of MALDI. By systematic and targeted variation of the functional groups of the α -cyanocinnamic acid core unit, 4-chloro- α -cyanocinnamic acid (Cl-CCA) was selected and synthesized, and it exhibited outstanding matrix properties. Key features are a substantial increase in sensitivity and a considerably enhanced peptide recovery in proteomic analyses because of a much more uniform response to peptides of different basicity. Using Cl-CCA as a matrix for a 1 fmol bovine serum albumin (BSA) in-solution digest, the sequence coverage is raised to 48%, compared with 4% for CHCA. For a gel band containing 25 fmol of BSA, unambiguous protein identification becomes possible with Cl-CCA. These findings also imply ion formation via a chemical ionization mechanism with proton transfer from a reactive protonated matrix species to the peptide analytes. The considerable increase in performance promises to have a strong impact on future analytical applications of MALDI, because current sensitivity limits are overcome and more comprehensive analyses come into reach.

ionization mechanism | proton transfer

Matrix-assisted laser desorption/ionization (MALDI) (1–3) relies on a cocrystallization of the analytes of interest with an excess of small organic compounds, the matrix, which is accomplished by either simply drying $\approx 1\text{-}\mu\text{l}$ droplet of a solution containing matrix and analyte (dried-droplet preparation) (2, 3) or by depositing a droplet of the analyte solution onto a prespotted matrix layer (surface preparation) (4). The matrix exhibits a strong absorption at the laser wavelength used (typically 337 nm or 355 nm in the UV), which, after irradiation with a short pulse of laser light, leads to the ablation of a shallow surface region into the vacuum of the mass spectrometer, the release of intact gaseous matrix and analyte molecules, and their partial ionization. Since its introduction in the 1980s, MALDI has found widespread use in the mass spectrometric analysis of biological macromolecules but recently also has been used increasingly for small-molecule analysis (5, 6).

However, the main field of application of MALDI mass spectrometry (MS) today is protein identification in proteomic schemes by using the peptide mass fingerprint (PMF) approach (7), often supplemented by MALDI-time-of-flight/time-of-flight (TOF/TOF) analyses. α -Cyano-4-hydroxycinnamic acid (CHCA) (8) as a matrix (in positive-ion mode) allows the researcher to identify proteins via MS or MS/MS analysis of their proteolytic peptide products (typically generated by trypsin) and by using protein or DNA sequence databases and dedicated search engines. This is accomplished directly from bands or spots of one- or two-dimensional (1D or 2D) sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) without laborious sample cleanup by harnessing the relatively high

tolerance of the MALDI technique for ubiquitous contaminants like salts and buffers. The conceptual simplicity of the technique lends itself to robotic sample preparation and to high-throughput analyses, in particular when combined with MALDI-TOF-type instruments. Detection limits strongly depend on the protein, but even in easy cases they are reached at the ≈ 50 fmol level. On the one hand, mass spectrometric instruments have constantly evolved over the last 15 years. However, despite numerous efforts to overcome apparent limitations of MALDI, such as the use of ionic liquids for spot heterogeneity (9) and matrix-free methods to avoid problems in the analysis of low mass analytes because of intense interfering matrix signals (10), CHCA used in dried-droplet or surface preparation mode still provides the “gold standard” for analytical proteomics by MALDI-MS. A major drawback of CHCA-MALDI is its pronounced preference for strongly basic arginine-containing peptides (11) and the concomitant suppression of small acidic peptides, which limits the sequence coverage and thus impedes the identification of scarce proteins.

Results and Discussion

The rationale of the approach followed here was to synthesize various matrix candidates by modifying the α -cyanocinnamic acid (CCA) core unit (Fig. 1) in the R₁- and R₂-positions. Each compound was inspected with regard to its mass spectrum and to its performance in a peptide mass fingerprinting experiment.

The results were as follows: (i) The free carboxylic function is essential for matrix performance in MALDI. It is hypothesized that this function is crucial for peptide incorporation into the matrix crystal lattice via ion-pair formation with positively charged peptide functional groups. (ii) All CCA derivatives exhibiting good matrix properties in the positive-ion mode show abundant ion signals of the protonated matrix. This abundance of protonated matrix ion signals points out their crucial role as chemical ionization reagents in the MALDI process via proton transfer to the analyte and to the important role of the gas-phase proton-affinity.

As a consequence of the above results, 4-chloro- α -cyanocinnamic acid (Cl-CCA) was synthesized because of its suspected lower proton affinity, and, indeed, a dramatic qualitative and quantitative improvement in MALDI performance was revealed.

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Conflict of interest statement: The invention of Cl-CCA as a MALDI matrix was filed as a German patent. The principle of tandem peptides was filed as a European patent.

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[†]T.W.J. and M.K. contributed equally to this work.

[¶]To whom correspondence should be addressed: E-mail: michael.karas@pharmchem.uni-frankfurt.de.

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Fig. 1. CCA core and different substituents of the synthesized CHCA derivatives.

The strong enhancement in MALDI performance is depicted for a bovine serum albumin (BSA) in-solution trypsin digest, which is a widely used reference sample. Fig. 2 shows the section of the m/z range 1,100–1,400 Da [the complete spectrum from 500 to 3,500 Da is shown in [supporting information \(SI\) Fig. S1](#)] for a total sample load of 1 fmol of BSA for Cl-CCA and CHCA (Fig. S1). Absolute peptide-ion intensities and signal-to-noise (S/N) ratios increase strongly when using Cl-CCA instead of CHCA; ion intensities increase by a factor of 2–75, and S/N ratios increase by an average factor of 22 for common peptides, with the only exception being the peptide RHPEYAVSVLLR. Besides the increase in ion intensities, the mass spectra change qualitatively by the appearance of substantially more peptide signals, many of which represent small and/or acidic peptides, including the C-terminal peptide LVVSTQTALA [MW = 1,001.6 Da, isoelectric point (IEP) = 5.52; IEP values were calculated with the Compute pI/Mw tool without consideration of posttranslational modifications (www.expasy.ch/tools/pi_tool.html)] (see [Fig. S1](#) and [Table S1](#)). For three independent experiments for each matrix applying stringent selection criteria (internal calibration and restriction to consistently observed signals within a mass tolerance of ± 20 ppm for CHCA and ± 10 ppm for Cl-CCA), only seven tryptic peptides were detected for the CHCA matrix as opposed to 34 tryptic peptides for the Cl-CCA matrix (without consideration of cystines). The sequence coverage for the 1 fmol sample was thus raised to 48% compared with 12% for CHCA [Table 1 (total number of detected peptides and sequence coverage) and [Tables S1 and S2](#) (detected peptides with Cl-CCA and CHCA, respectively)]. For

a sample containing 10 fmol of BSA digest, the sequence coverage was 40% in the case of CHCA, compared with 75% for Cl-CCA.

Because quantitative data on the preference for arginine peptides or the suppression of lysine-containing and acidic peptides cannot be deduced from a BSA digest, this issue was addressed in a further experiment. Three synthesized “tandem” peptides with the amino acid sequences ITITPNRITITPNK (I), ITITPNRITITPNV (II), and ITITPNKITITPNV (III) were each subjected to trypsin digestion, which resulted in three binary mixtures of equimolar concentration of the two cleavage peptides. Fig. 3 shows a comparison of the MALDI mass spectra obtained for peptide (II) directly out of the reaction vial after dilution to 1 μM , using Cl-CCA (Fig. 3A) and CHCA (Fig. 3B) matrices. Although the signal of the peptide ITITPNV is at the detection limit (S/N ratio of 2) in the case of CHCA, its relative abundance is 34% compared with the ITITPNR signal for the Cl-CCA matrix (see Fig. 3 magnifications). Summarizing all three experiments, the signal-intensity ratio ITITPNR:ITITPNK:ITITPNV amounts to 100:8.4:0.22 for CHCA and to 100:56:34 for the Cl-CCA matrix.

The much lower discrimination effects for peptides were also corroborated in the analysis of phosphopeptides. An in-solution trypsin digestion of β -casein (containing <10% α -casein) served as an example. Samples of digested protein (50 fmol) were spotted with CHCA and Cl-CCA respectively. Analyzing the sum spectra of five independent mass spectra consisting of 500 shots each, only two phosphorylated α -/ β -casein peptides with an average intensity of 57 absolute counts and an average S/N ratio of 15 (monoisotopic peptide signals) could be detected using CHCA. In the case of Cl-CCA, eight phosphorylated α -/ β -casein peptides were detected with an average intensity of 975 absolute counts and an average S/N ratio of 131 (monoisotopic peptide signals) ([Table S3](#) and [Fig. S2](#)).

In a further set of experiments, the two matrices, CHCA and Cl-CCA, were inspected with regard to their dynamic range of peptide detection by adding an increasing molar excess (100- to 10,000-fold) of the strongly basic peptide bradykinin (RPPGF-SPFR) to a 10 fmol/ μL BSA digest. Again, Cl-CCA proved to be highly superior, because even at a 1,000-fold excess of bradyki-

Fig. 2. MALDI mass spectra of a tryptic in-solution digest of BSA using Cl-CCA (A) or CHCA (B) as a matrix. Mass numbers of assigned BSA peptides are given only for those signals occurring in three independent mass spectra within ± 20 ppm; m/z section 1,100–1,400 Da; total sample load, 1 fmol.

To a first view, it may appear surprising that already a small reduction in the PA can account for the strong effects observed. PAs of the basic functional groups of peptides are substantially larger than those of all matrices in use today for peptide or

Attempts to further decrease the PA of the matrix by more or stronger electron-withdrawing substituents failed because of the induced hypsochromic shifts and the reduction of the absorption at the wavelength of 337 nm used for MALDI. Also, the absorption spectrum of Cl-CCA exhibits a strong hypsochromic shift compared with the one of CHCA; therefore, the shown strong increase in performance is only achievable in 337-nm instruments (CHCA: $\epsilon(337 \text{ nm}) = 17.600$, $\epsilon(355 \text{ nm}) = 7.100$; Cl-CCA: $\epsilon(337 \text{ nm}) = 3.580$, $\epsilon(355 \text{ nm}) = 70$) (Fig. S7). The replacement of the carboxylic group by sulfonic or phosphonic acids failed for the same reason. Vice versa, the introduction of additional functional groups to yield a bathochromic shift was not successful, because in these cases an undesired stabilization

Matrix	Min 1 of 3 spectra error <80 ppm, peptides (% coverage)	Min 2 of 3 spectra error <40 ppm, peptides (% coverage)	All 3 spectra error <30 ppm, peptides (% coverage)	All 3 spectra error <20 ppm, peptides (% coverage)	All 3 spectra error <15 ppm, peptides (% coverage)	All 3 spectra error <10 ppm, peptides (% coverage)
CI-CCA	35 (46)	24 (35)	17 (25)	14 (22)	9 (14)	5 (7)
CHCA	6 (14)	– (0)	– (0)	– (0)	– (0)	– (0)

of the protonated matrix species and thus enhancement of the PA was promoted at the same time. Moreover, a second important property of a high-performance matrix lies in the formation of compact crystals, which is not predictable. This allows one to use low matrix concentrations and results in the accumulation of analytes in these matrix crystals. Already minor chemical changes, such as the addition of a methyl group to the aromatic ring, may totally change the crystallization. Compared with other halogenated cyanocinnamic acid derivatives, only Cl-CCA has this beneficial property.

Materials and Methods

Materials and Reagents. All chemicals were purchased from Sigma Aldrich and were analytical grade, except modified trypsin, which was proteomics grade, and when otherwise noted. The in-solution BSA digest (Starter Kit for MALDI-TOF MS, Part-No. 208241, Lot 2004–208241-001) and CHCA (Part-No. 201344, Lot 2007–201344-001) were purchased from Bruker Daltonics. The strong cation exchanger was from Merck (Ionenaustauscher I, pro analysis); vanillin (99%), 2-chloro-4-fluorobenzaldehyde (97%), and methanol (HPLC-grade) were from Acros. Acetonitrile (ACN) was Rotisolv HPLC-Grade and purchased from Roth, and 4-fluoro-3-methoxybenzaldehyde (98%) was acquired from ABCR. MilliQ water was prepared freshly in-house. The synthetic peptides ITITPNRITITPNK, ITITPNKITITPNV, and ITITPNRITITPNV were obtained from R. Pipkorn [Central Peptide Synthesis Unit, German Cancer Research Center (DKFZ)].

Synthesis of Substituted CCAs. All CCA derivatives were synthesized by a standard Knoevenagel condensation using cyanoacetic acid or its derivatives and substituted benzaldehydes. Ammonium acetate was used as a catalyst. In a typical approach, 2 g of cyanoacetic acid (1 eq), 0.9 eq of the benzaldehyde, and 0.15 eq of ammonium acetate were refluxed with stirring in sufficient amounts of toluene (~50 ml) for complete dissolving. After quantitative separation of the reaction water by a water separator (~3 h), the reaction mixture was cooled to ~50°C and filtered. The crude product was washed with sufficient amounts of distilled water and purified by repeated recrystallization from methanol/water (1:1). Cl-CCA was purified further by using cation-exchange columns followed by additional recrystallization from ACN/water. Overall yields were between 70 and 90%. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.66 (d, 2H, J = 9.4 Hz), 8.02 (d, 2H, J = 9.8 Hz), 8.33 (s, 1H). Accurate mass determination: (M+H)⁺ 208.01718 ± 0.00030 Da (error: 5.7 ± 1.5 ppm), Calc. 208.01598 Da.

One-Dimensional SDS-PAGE Electrophoresis. One-dimensional gel electrophoresis was performed by using standard methods (20) on the Bio-Rad MiniProtein 3 Cell system (8 × 7.3 cm minigels). Ten-percent acrylamide gels of 0.75-mm thickness were used. BSA was dissolved in sample buffer to a final concentration of 25 fmol/15 μl, loaded into the wells, and subjected to electrophoresis using 50 mA per slab for 1–2 h. Gels were subsequently fixed and silver stained by using standard methods (21).

In-Gel Digestion. Protein bands obtained after the application of 25 fmol of BSA to a 1D gel were faintly visible after silver staining. After in-gel digestion overnight at 37°C with trypsin in 20 μl of 10 mM aqueous ammonium hydrogencarbonate buffer, according to standard procedures (22), the combined extraction solutions were dried at 40°C and 35 mbar.

In-Solution Digestion. Tryptic in-solution digestion of β-casein from bovine milk (≥90% β-casein) was performed by using standard conditions with 25 mM ammonium bicarbonate buffer solution.

Digestion of Synthetic Peptides ITITPNRITITPNK, ITITPNRITITPNV, and ITITPNK-ITITPNV. A 10-μl sample of each peptide solution (1 mM) was treated with 9 μl of aqueous ammonium hydrogencarbonate (50 mM) and 1 μl of trypsin-solution (1 μg/μl) and digested overnight at room temperature. The resulting stock solution (500 pmol of digested peptides per μl) was diluted with 30% ACN/0.1% trifluoroacetic acid (TFA) to a final concentration of 1 μM and directly used for MALDI analysis.

Sample Preparation and Measurement. A mixture of 0.5 μl of matrix solution (30 mM, 70% ACN) and 1 μl of BSA in-solution digest (diluted to 1 nM, 30% ACN, 0.01% TFA), 1 μl of the synthetic peptide digest, or 1 μl of the β-casein in-solution digest (diluted to 50 nM, 30% ACN, 0.1% TFA) were spotted without premixing onto a polished stainless steel target and air dried.

The 25 fmol/band BSA in-gel digest sample was redissolved in 4 μl of 30% ACN/0.01% TFA water. Then 2 μl of the analyte solution was spotted onto a polished steel target and mixed directly onto the plate, either with 0.5 μl of 30 mM CHCA or Cl-CCA in 70/30 ACN/1.5% TFA water. Neither CHCA nor Cl-CCA crystals were washed after air drying. NH₄H₂PO₄ (aqueous, V = 1 μl, c = 10 mM) was added for suppression of matrix cluster ions (23).

Mass Spectrometry. All MS measurements were carried out with an Applied Biosystems Voyager DE-STR TOF mass spectrometer with a 337-nm nitrogen laser at laser fluences optimized for the highest analyte S/N ratio. Mass spectra shown for BSA digests were accumulated from 500 single laser shots. Mass spectra shown for the β-casein digest were accumulated from five single mass spectra with 500 laser shots each. Data Explorer 4.5C was used for analysis of the spectra. Peak detection of phosphopeptides was performed by using a S/N threshold of 10 after internal calibration and advanced baseline correction with preset parameters.

Intensity ratios for cleaved tandem peptides are the average of six measurements with 500 laser shots per mass spectrum. MALDI-TOF parameters are polarity, positive; operation mode, reflector; accelerating voltage, 20 kV; grid voltage, 68.5%; delay time, 150 ns; shots per spectrum, 500; mass range, 500–3,500 Da; low mass gate, 450 Da; bin size, 0.5 ns; minimum S/N ratio for peptide detection of in-solution digested BSA, 4 [in the case of in-gel digestion samples S/N > 35 (800–1,000 Da), 15 (1,000–1,300 Da), 12 (1,300–1,500 Da), 8 (1,500–3,000 Da)]. Accurate mass determination of [Cl-CCA+H]⁺ was performed with a mixture of Cl-CCA and CHCA at a ratio of 1:100 (n/n) by using a Voyager DE-STR. The mass of [Cl-CCA+H]⁺ was calculated as the average value of eight measurements with 500 shots each. Ion peaks of CHCA⁺⁺ and [2CHCA+H]⁺ were used as internal calibrants.

PA Determination of Cl-CCA. Determination of the PA value of Cl-CCA was carried out by postsource-decay fragmentation (24) of the proton-bound heterodimers (17, 19, 25) of CHCA and Cl-CCA by using different fluences, followed by a measurement of the peak area ratios of the resulting protonated matrix monomers (Fig. S8). The laser-fluence-dependent effective temperature, *T*_{eff}, was detected by using the thermometer molecule *N*-*p*-methoxybenzylpyridinium (M⁺) chloride, and the MS/MS measurements were recorded at the same fluence (Fig. S9). Thermometer molecules yield only one intense fragment F⁺ and enable a correlation between the experimental survival yields SY = I(M⁺)/I(M⁺) + I(F⁺) and *T*_{eff} by calculated dissociation rate constants from Rice-Ramsperger-Kassel-Marcus models (26, 27) (Fig. S10). To eliminate temperature influences caused by CHCA, the Cl-CCA:CHCA ratio was chosen at 10,000:1 (n/n), which was the highest possible ratio with the formation of intense heterodimer ions. By using CHCA as a reference base with a known PA, determination of the PA of Cl-CCA was possible by the simplified expression of the kinetic method:

$$\ln \frac{k_{\text{CHCA}}}{k_{\text{Cl-CCA}}} = \ln \frac{[\text{CHCAH}^+]}{[\text{Cl-CCAH}^+]} = \frac{\text{PA}_{\text{CHCA}} - \text{PA}_{\text{Cl-CCA}}}{RT_{\text{eff}}} \quad [2]$$

The chosen parameters for the MS/MS measurements were as follows: laser fluence, 2,200–2,800 units, step 100; polarity, positive; operation mode, reflector; accelerating voltage, 20 kV; grid voltage, 68.5%; delay time, 150 ns; shots per segment, 500; mirror ratios, 1, 0.6, and 0.45; spectrum processing, Gaussian smooth (25 points). The mirror ratios for the segment spectra were chosen in such a way that the protonated matrix monomers were recorded together in one segment.

The effective temperatures and the correlating matrix areas for the different laser fluences used are listed in Table S5.

Computational Methodology. Visualization and initial building were done by using ChemDraw Pro 10.0 and Chem3D Ultra 10.0 running on a commercial PC system (C2D E6600, 2GB RAM). Initial geometries of the neutral and protonated compounds were constructed and optimized energetically by a slightly modified Allinger MM2 force field with a root-mean-square gradient of 0.1. For further optimization of the geometries, DFT calculations using the hybrid B3LYP method and 6–311G** basis set (28) were performed with the Gaussian03 implementation of DFT.

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