Mass Spectrometry of Proteins

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Discrimination and Selective Enhancement of Signals in the MALDI Mass Spectrum of a Protein by Combining a Matrix-Based Label for Lysine Residues with a Neutral Matrix

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To understand or control the function of a protein, it is necessary to determine its three-dimensional structure. Despite the automation of some steps in the experimental determination of 3D structures by X-ray crystallography or NMR spectroscopy, a number of bottlenecks still exist in these processes. For this reason, alternative approaches, such as methods based on mass spectrometry (MS), have been used to obtain information on the structure and interactions of proteins.[1,2] Some of these approaches involve chemical modifications or the cross-linking of amino acid side chains. Chemical modifications can be used to study the solvent accessibility or reactivity of specific side chains.[3–5] For example, proteins containing lysine residues have been treated with *N*-hydroxysuccinimide (NHS) esters derived from a variety of compounds. NHS esters react with the accessible side-chain amino groups of the protein. To avoid disturbing the protein structure, the degree of modification should be limited.

After proteolysis, trying to find modified peptides in a mixture that contains unmodified peptides in greater abundance is like looking for a needle in a haystack. Attempts have been made to enrich the mixture in modified peptides by using reagents with high-affinity handles, such as biotin labels and avidin extraction columns.[6,7] Another approach consists of the use of isotope-labeled reagents to give a characteristic mass-spectrometric pattern, which helps in the identification of the modified peptides.[8,9] Several derivatization-based strategies to improve the analysis of peptides by enhancement of the MALDI MS signal intensities have been described for UV MALDI ionization, including the use of coumarin tags.[10] In these technologies, the matrix absorbs the laser UV light and transmits energy to the directly linked protein or peptide molecules, thus promoting their desorption and ionization.[11,12]

We report herein a new approach in which peptides are labeled with α-cyano-4-hydroxycinnamic acid (HCCA), which absorbs UV light, to improve their identification through the relative enhancement and discrimination of the MALDI MS signal. When the lysine residues in cytochrome c were labeled in this way, it was possible to discriminate labeled peptides of interest that were present in low concentration from unlabeled more abundant peptides after hydrolysis of the protein.

We first synthesized the model peptide (HCCA)–RKNGPLIGAF–NH_2, in which HCCA has been coupled to the N terminus of the peptide chain (see the Experimental Section). This labeled peptide was mixed with the unlabeled form of the peptide and with an endoproteinase Lys-C lysate of horse-heart cytochrome c in a molar ratio of 1:1:23. We used the classical HCCA matrix to observe the different proteolytic peptides. The peak at *m/z* 1242 corresponding to the labeled peptide had a comparable intensity to those of the peptides from the lysate and was six times as intense as the peak corresponding to the unlabeled form (*m/z* 1071; see the Supporting Information). When the neutral matrix α-cyano-4-hydroxycinnamic methyl ester (HCCE) was used, the major peak was that of the labeled peptide in spite of its relatively low abundance, whereas no peak corresponding to the unlabeled peptide was visible (see the Supporting Information). The labeling of a peptide with an HCCA moiety can thus increase its response in the acidic matrix. However, the neutral matrix was found to discriminate its signal much more effectively from those of the unlabeled form and the peptides from the lysate. A combination of the neutral matrix and a UV-light-absorbing label bound to the peptide can thus help in the identification of a specific peptide in a mixture by enhancing relatively its MALDI mass spectral response.

Next, we applied this methodology to the MALDI MS analysis of a protein lysate to discriminate labeled peptide fragments. The first step was to synthesize the NHS ester of

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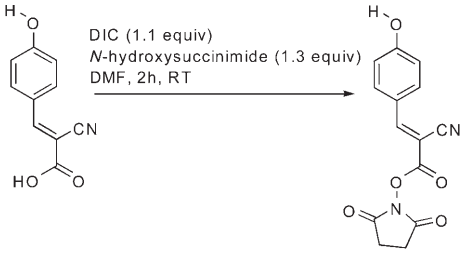

Scheme 1. Synthesis of HCCA–NHS. DIC = diisopropylcarbodiimide, DMF = *N*,*N*-dimethylformamide.

HCCA (Scheme 1) with the aim of labeling the accessible lysine residues in a protein. This reagent, HCCA–NHS was then mixed with cytochrome c in a reagent/lysine molar ratio of 4:1 (that is, in a reagent/cytochrome c molar ratio of 76:1, as cytochrome c contains 19 lysine residues). The MALDI mass spectrum obtained of the modified cytochrome c with a sinapinic acid matrix showed a mixture of species that correspond to cytochrome c with 0–4 adducts; the peak of greatest intensity was due to cytochrome c with one adduct (see the Supporting Information). The mass shifts are in agreement with the expected value of 171 Da, which corresponds to the acylation of a lysine side chain by one HCCA–NHS molecule.

Cytochrome c that had been modified by treatment for 5 min with HCCA–NHS in a reagent/lysine molar ratio of 4:1 was then digested with endoproteinase Lys-C. The lysate was analyzed by MALDI MS by using the classical HCCA matrix (Figure 1). The peaks that had been observed with unmodified cytochrome c were also observed with this lysate. New peaks corresponding to labeled peptides were rather small and were limited to fragments with the sequences 26–39 (*m*/*z* 1733) and 28–53 (*m*/*z* 2920), which contain the labeled residues Lys27 and Lys39, respectively. This limited number of modified peptides at low concentration probably reflects the limited degree of labeling of cytochrome c. In contrast, when the neutral matrix HCCE was used, the only peak observed in the mass spectrum (Figure 2) that was also present in the mass spectrum of unmodified cytochrome c was that corresponding to the sequence with the heme moiety (sequence 14–22, *m*/*z* 1634). This peak was the major peak in the lysate of unmodified cytochrome c. All other peaks corresponded to labeled peptides; their intensities had been enhanced in a relative sense by the association of the HCCA-derived label with the neutral matrix.

Whereas other labeling tags can enhance the intensities of peptide peaks in MALDI MS more than 40-fold,[10] the intensity of the peaks is not strongly increased with our method. However, the combination of a HCCA-derived label with HCCE matrix allowed the selective discrimination of the labeled peptides. Each peak could be identified as corresponding to a peptide fragment with one or two modified lysine residues (Table 1) by taking into account the mass shifts of 171 or 342 Da induced by the chemical modifications.

When the Getarea software was used (http://pauli.utmb.edu/getarea/), no lysine residue appeared to be buried, which indicates that the majority of lysine residues were labeled. Most of the unmodified lysine residues were also

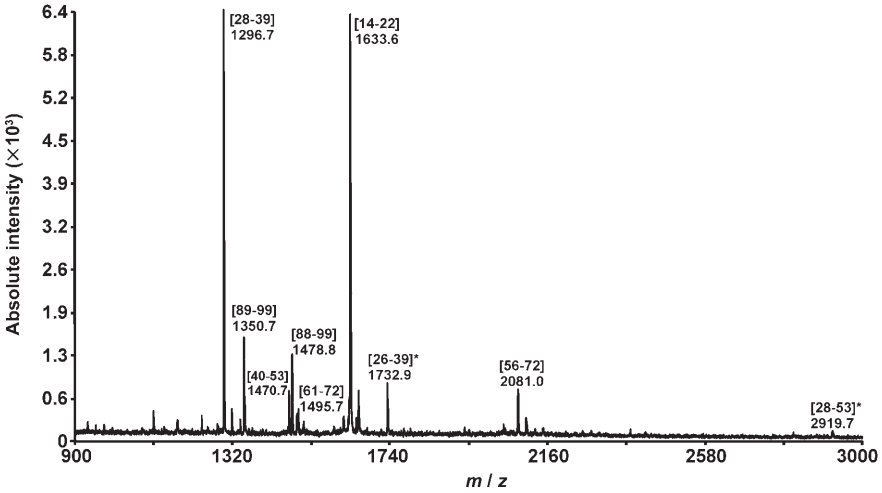

Figure 1. MALDI mass spectrum obtained with the acidic matrix HCCA of peptides (500 fmol) generated by the digestion with endoproteinase Lys-C of cytochrome c labeled with HCCA–NHS. Peaks with an asterix are of labeled fragments.

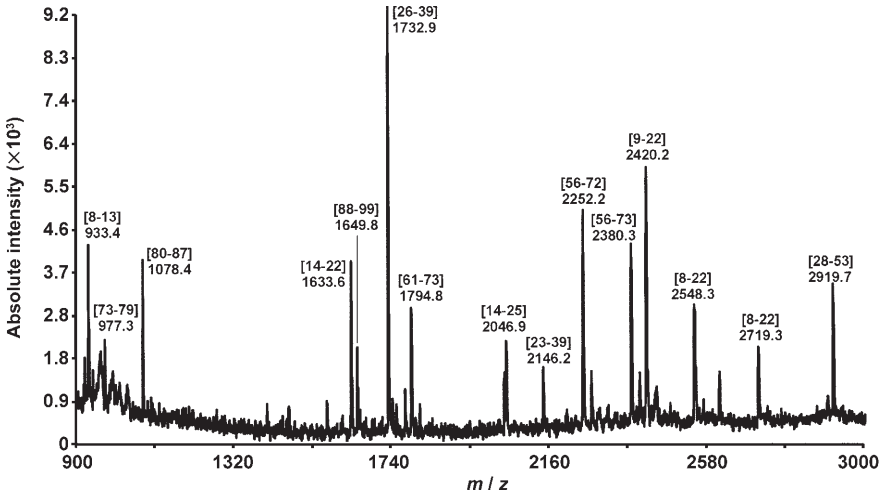

Figure 2. MALDI mass spectrum obtained with the neutral matrix HCCE of peptides (500 fmol) generated by the digestion with endoproteinase Lys-C of cytochrome c labeled with HCCA–NHS. Sequence [14–22], *m*/*z* 1634 is the only peak that was also present in the mass spectrum of unmodified cytochrome c.

Table 1: Measured and calculated masses (asterixes indicate the ions that contain the heme) that enable the identification of peptides with labeled lysine residues (peaks from Figure 2).

$[M+H]^+$ or M^+(*) experimental	calculated	Sequence	Lys	$[M+H]^+$ or M^+(*) experimental	calculated	Sequence	Lys
933.4	933.5	8–13	8	2146.2	2146.1	23–39	25 and 27
977.3	977.5	73–79	73	2252.2	2252.2	56–72	60
1078.4	1078.6	80–87	86	2380.3	2380.1	56–73	60 or 72
1649.8	1649.9	88–99 or 89–100	88 or 99	2420.2*	2420.0*	9–22 (with heme)	13
1732.9	1732.9	26–39	27	2548.3*	2548.1*	8–22 (with heme)	8 or 13
1794.8	1794.8	61–73	72	2719.3*	2719.1*	8–22 (with heme)	8 and 13
2046.9*	2046.8*	14–25 (with heme)	22	2919.7	2919.4	28–53	39

found to be unreactive in a number of cross-linking studies with cytochrome c and NHS or imidoester cross-linkers.[8,13,14] This absence of reactivity might be explained by the unfavorable environment of these lysine residues.

In summary, we synthesized an *N*-hydroxysuccinimide ester of HCCA, a matrix used in MALDI MS, and used it to label the lysine side chains of cytochrome c. When the HCCA matrix was used for MALDI MS of the lysate of the resulting modified cytochrome c, the intensities of the signals observed for the acidified peptides (labeled and unlabeled) were proportional to the relative abundance of the peptides. Therefore, very few labeled peptides were distinguished. In contrast, when the neutral matrix HCCE was used under neutral conditions, the global signal intensity decreased as a result of less favorable protonation conditions. However, the desorption of the labeled peptides was facilitated by the bound HCCA group (which absorbs the laser energy), and the signals for these peptides appeared more intense than the signals corresponding to the unlabeled peptides. This approach can be used to label lysine residues specifically, thereby decreasing the risk of disturbing the protein structure. It can also be used more generally to increase significantly the sensitivity of the detection of labeled compounds, including peptides, by MALDI MS.

Experimental Section

Synthesis of the model labeled peptide (HCCA)-RKNGPLIGAF-NH_2: The Fmoc (9-fluorenylmethoxycarbonyl) solid-phase strategy was used for peptide synthesis. After anchoring the first residue on Rink-amide-functionalized polystyrene-resin beads, deprotection and coupling steps were carried out by using standard protocols until the desired sequence had been synthesized. (*O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and *N*-methylmorpholine (NMM) were used for the coupling steps, DMF/piperidine for the deprotection steps.) HCCA was activated with HBTU/NMM and coupled to the N terminus of the peptide. The resulting peptide labeled at the N terminus was cleaved from the resin with trifluoroacetic acid/water/triisopropylsilane (95:2.5:2.5, v/v/v). Following the removal of the resin by filtration, the cleavage cocktail was removed by vacuum centrifugation. The model labeled peptide was precipitated with diethyl ether, the solvent removed by filtration, an acetonitrile/water 50:50 (v/v) mixture was added to solubilize the peptide, and the mixture was freeze dried.

Synthesis of HCCA–NHS: A solution of *N*-hydroxysuccinimide (115 mg, 2.3 mmol) in DMF (10 mL) was added to a solution of HCCA (334 mg, 1.77 mmol) and diisopropylcarbodiimide (298 μL, 1.3 mmol) in DMF (20 mL), and the resulting mixture was stirred for 2 h. The mixture was then concentrated to dryness in vacuo, and the residue was purified by normal-phase chromatography (CH_2Cl_2/MeOH 98:2) to give HCCA–NHS (468 mg, 1.63 mmol, 93%) as a pale yellow solid. ^{1}H NMR ([D_6]DMSO, 300 MHz): $\delta = 8.45$ (s, 1H), 8.07 (d, $J = 9$ Hz, 2H), 6.96 (d, $J = 9$ Hz, 2H), 2.83 ppm (s, 4H); ^{13}C NMR ([D_6]DMSO, 75 MHz): $\delta = 169.9$ (2C), 164.5 (1C), 159.5 (1C), 159.1 (1C), 135.5 (2C), 122.4 (1C), 116.7 (2C), 115.1 (1C), 90.5 (1C), 25.5 ppm (2C).

Cytochrome c labeling with HCCA–NHS and proteolysis: A solution of HCCA–NHS in dimethyl sulfoxide (14 mM, 5 μL) was incubated at room temperature for 5 min with a mixture of cytochrome c in a phosphate buffer (10 μM in cytochrome c, 20 mM in phosphate, 150 mM in NaCl, pH 7.5, 95 μL). (These quantities correspond to an HCCA–NHS/protein ratio of 76:1.) The reaction was then quenched with Tris buffer (3 M; Tris = tris(hydroxymethyl)aminomethane). The resulting mixture was diluted with water, and the modified protein was digested with endoproteinase Lys-C (protease/protein 1:20, w/w) for 18 h at room temperature.

MALDI mass spectrometry: The MALDI mass spectra were acquired with an Applied Biosystems Voyager XL instrument. Labeled cytochrome c (1 μM, 0.5 μL) was mixed on the MALDI probe with the sinapinic acid matrix (saturated in acetonitrile/water/trifluoroacetic acid 50:50:0.3, 0.5 μL). When the HCCA matrix was used, the peptide solution (0.5 μL) was mixed on the MALDI probe with the matrix solution (half-saturated in acetonitrile/water/trifluoroacetic acid 50:50:0.3, 0.5 μL). When the HCCE matrix was used, the matrix solution (saturated in acetone, 0.5 μL) was deposited and dried on the MALDI probe, and the peptide solution (0.5 μL) was then deposited.

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