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Matrix-assisted laser desorption mass spectrometric peptide mapping of proteins separated by two-dimensional gel electrophoresis: Determination of phosphorylation in synapsin I

WENZHU ZHANG,[1] ANDREW J. CZERNIK,[1] TOM YUNGWIRTH,[2] RUEDI AEBERSOLD,[2,3] AND BRIAN T. CHAIT[1]

[1] Laboratory for Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, New York 10021
[2] The Biomedical Research Center and Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

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Abstract

A technique is described for the rapid, sensitive analysis of posttranslational modifications of proteins that have been separated by 2-dimensional electrophoresis and blotted onto a membrane with a cationic surface. The isolated protein spots visualized by reverse staining of the blotting membrane are excised, washed, and subjected to chemical (cyanogen bromide) and/or enzymatic (endoproteinase Lys-C) degradation directly on the membrane. The resulting mixture of peptide fragments is extracted from the membrane into a solution that is compatible with matrix-assisted laser desorption mass spectrometric analysis and analyzed without fractionation. Relatively accurate (±1 Da) mass determination of these peptide fragments provides a facile and sensitive means for detecting the presence of modifications and for correlating such modifications with the differential mobility of different isoforms of a given protein during 2-dimensional electrophoresis. The technique is applied to the determination of sites of phosphorylation in synapsins Ia and Ib, neuronal phosphoproteins that are believed to function in the regulation of neurotransmitter release and are substrates for cAMP and Ca^{2+}/calmodulin-dependent protein kinases, which appear to control their biological activity.

Keywords: mass spectrometry; matrix-assisted laser desorption ionization; peptide map; protein phosphorylation; synapsin I; 2D PAGE

Whereas technology for the determination of amino acid sequences of proteins has been highly developed (Stolowitz, 1993), methods for the localization and characterization of posttranslationally modified amino acid residues are considerably less evolved. This deficiency is particularly significant, for example, in investigations of cellular regulatory pathways, which depend on the modulation of enzyme activities or stabilization of functional enzyme complexes by reversible protein phosphorylation (Hunter & Sefton, 1991; Pawson & Gish, 1992).

Protein isoforms, including differentially phosphorylated proteins, can sometimes be resolved using high-resolution analytical techniques such as 2-dimensional polyacrylamide gel electrophoresis. Two-dimensional PAGE incorporates isoelectric focusing in the first dimension and SDS-PAGE in the second dimension (O'Farrell, 1975). Because of the high selectivity of IEF for small charge differences, 2DE is particularly well suited for the separation of differentially phosphorylated proteins. However, 2DE is a descriptive technique that does not explicitly reveal the structural basis for differences in the mobility of the resolved proteins.

With the advent of sensitive and versatile new methods for ionizing peptides and proteins, mass spectrometry is rapidly becoming a method of choice for the characterization of posttranslational modifications (Chait & Kent, 1992; Aebersold, 1993).

Reprint requests to: Brian T. Chait, Laboratory for Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10021; e-mail: chait@rockvax.rockefeller.edu.

[3] Present address: Department of Molecular Biotechnology, University of Washington FJ-20, Seattle, Washington 98185.

Abbreviations: 1DE, 1-dimensional polyacrylamide gel electrophoresis; 2DE, 2-dimensional polyacrylamide gel electrophoresis; IEF, isoelectric focusing; MS, mass spectrometry; MW, molecular weight; MALDI, matrix-assisted laser desorption/ionization; HFIP, hexafluoroisopropanol; 4-HCCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; ACN, acetonitrile; NEPHGE, nonequilibrium pH gel electrophoresis; T, total acrylamide concentration, C, bis-acrylamide crosslinker concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CaM, calcium/calmodulin-dependent; Lys-C, endoproteinase Lys-C.

Techniques such as matrix-assisted laser desorption/ionization (Hillenkamp et al., 1991) and electrospray ionization (Fenn et al., 1989) routinely allow the mass analysis of peptides and proteins with accuracies that are often sufficient for the detection of low molecular weight modifications in the intact molecule. For a more detailed elucidation of the sites and nature of modifications in proteins, mass spectrometric measurements are made on peptides generated by targeted chemical or enzymatic fragmentations of the proteins (Biemann, 1992; Roepstorff & Hojrup, 1993). However, the practical value of MS for the structural characterization of protein isoforms has been limited because polypeptide samples separated by 2DE have not been readily compatible with MS. Nevertheless, a number of mass spectrometric investigations have been undertaken of electrophoretically separated proteins (Eckerskorn et al., 1992; Roepstorff & Klarskov, 1993; Strupat et al., 1994) and peptides derived from these separated proteins by enzymatic (usually tryptic) digestion (Camilleri et al., 1989; Yates et al., 1989; Hall et al., 1992, 1993; Daga et al., 1993; Geromanos et al., 1993; Henzel et al., 1993; Hess et al., 1993; Mahrenholz et al., 1993; Wang et al., 1993; Winz et al., 1993; Yates et al., 1993). These measurements (usually of polypeptides eluted from blotting membranes) have been made with a variety of different mass spectrometric techniques, including secondary ionization MS (Camilleri et al., 1989; Yates et al., 1989; Hall et al., 1992, 1993; Mahrenholz et al., 1993), plasma desorption MS (Roepstorff & Klarskov, 1993), electrospray ionization MS (Daga et al., 1993; Hess et al., 1993; Winz et al., 1993, Yates et al., 1993), and MALDI-MS (Eckerskorn et al., 1992; Geromanos et al., 1993; Henzel et al., 1993; Strupat et al., 1994; Wang et al., 1993). With only a single exception (Henzel et al., 1993), measurement of the proteolytic peptides was performed subsequent to HPLC fractionation of the mixture of peptides.

In the present study, we demonstrate the application of MALDI time-of-flight MS to the analysis of proteins that have been separated by 2DE and electroblotted onto a membrane with a cationic surface. In particular, we used the technique to examine protein phosphorylation. The isolated proteins were subjected to chemical and/or enzymatic degradation directly on the membrane, and the resulting unfractionated mixture of peptide fragments was extracted from the membrane into a solution that was compatible with analysis by MALDI-MS. Accurate mass determination of these peptide fragments provides a facile and sensitive means for detecting the presence of modifications and for correlating such modifications with the differential mobility of different isoforms of a given protein during 2DE. We have applied the technique to the determination of sites of phosphorylation in synapsins Ia and Ib, neuronal phosphoproteins that are believed to function in the regulation of neurotransmitter release (Valtorta et al., 1992; Greengard et al., 1993) and are substrates for cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases, which appear to control their biological activity.

Results and discussion

The strategy employed for mapping peptides from proteins separated by 2DE and electroblotted onto Immobilon CD membrane is outlined in Figure 1. Protein spots visualized by reverse staining of the blotting membrane were excised, washed, and subjected to cyanogen bromide and/or enzymatic digestion using endoproteinase Lys-C. The resulting mixture of peptide fragments was extracted from the membrane, combined with a laser desorption matrix, and analyzed, without fractionation, by MALDI-MS.

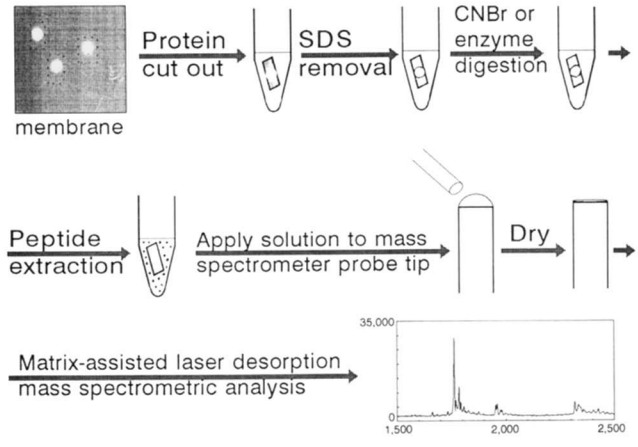

Fig. 1. Strategy employed for the mass spectrometric mapping of peptides generated from proteins electroblotted onto Immobilon CD membranes from 2-dimensional polyacrylamide electrophoretic gels.

Peptide mapping of horse skeletal muscle myoglobin

To establish appropriate conditions for the digestion of proteins separated by 1DE or 2DE and the subsequent extraction and analysis of the resulting peptides by MALDI-MS, a series of studies was carried out on horse skeletal myoglobin (MW = 16,951 Da). Complete CNBr cleavage of horse skeletal myoglobin (which contains 2 methionine residues) produces 3 peptide fragments, designated a, b, and c with MWs of 6,216 Da, 8,162 Da, and 2,513 Da, respectively (assuming homoserine lactone residues at the C-termini). Incomplete cleavage of myoglobin at the methionine residues generates the additional fragments bc (MW = 10,705 Da) and ab (MW = 14,409 Da).

Figure 2 shows MALDI mass spectra of myoglobin-derived CNBr fragments extracted from the membrane under 2 different conditions. The first extraction (Fig. 2A) was carried out by adding 40 μL of α-cyano-4-hydroxycinnamic acid (30 mM) in 2.5% aqueous TFA:30% ACN (v/v) to the membrane. A 0.5-μL aliquot of this extraction/matrix solution was applied to the sample probe tip and analyzed. The second extraction (Fig. 2B) was carried out on a separate membrane using 20 μL of a solution (30 mM 4-HCCA in 2.5% aqueous TFA:60% ACN [v/v]) containing a larger proportion of organic solvent than was used in the first extraction. Comparison of the spectra in Figure 2A and B indicates significant differences in the extraction/solubilization efficiencies of these 2 solutions. The dominance of peak c in Figure 2A indicates that this low molecular weight carboxyl-terminal fragment was extracted/solubilized efficiently by the first, more polar solution, while the much less intense peaks corresponding to the larger fragments a, b, ab, and bc and intact apomyoglobin, M, indicate that these larger peptides were released with reduced efficiency from the membrane. These larger peptides were solubilized and released from the membrane with improved efficiency by the solution containing the higher proportion of organic solvent (Fig. 2B). Improved extraction of the larger fragments and intact apomyoglobin was also achieved using a solution of 15 mM 4-HCCA in 50% hexafluoroisopro-

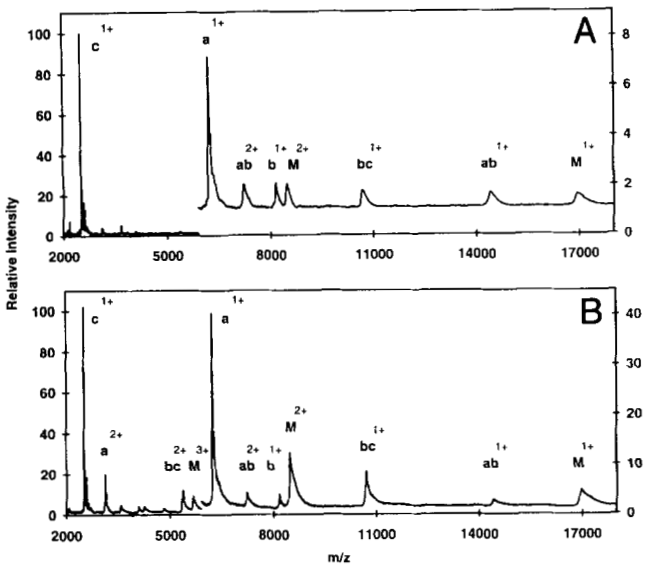

Fig. 2. Matrix-assisted laser desorption/ionization mass spectra of the products of CNBr digestion of equine myoglobin obtained from Immobilon CD membranes. Experiments were performed using 2 different extraction solutions: **A**, 30 mM 4-HCCA in 2.5% aqueous TFA:30% ACN (v/v); **B**, 30 mM 4-HCCA in 2.5% aqueous TFA:60% ACN (v/v). The scale on the right-hand axis refers to the portion of the spectrum that has been expanded vertically. a, b, and c denote fragments produced by CNBr. ab and bc are products resulting from incomplete cleavage by CNBr. M denotes intact myoglobin. The numbers adjacent to the peaks indicate the number of protons attached to the neutral peptides. The low-intensity unlabeled peaks between *m/z* 3,200 and 5,000 correspond to ab^{3+}, bc^{3+}, M^{4+}, and ab^{3+}, respectively.

panol:1.25% aqueous TFA:15% ACN (v/v/v) (data not shown). The experimentally determined molecular weights of the CNBr fragments a, b, c, and bc were in accord with the calculated molecular weights to better than 1 Da. By contrast, the measured molecular weights of the larger fragment ab (MW = 14,409 Da) and intact apomyoglobin (MW = 16,951 Da) were typically 20–50 Da in excess of the calculated values. These discrepancies are an order of magnitude larger than normally observed for apomyoglobin that is not derived from a blotting membrane after gel electrophoretic separation. Although the source of these discrepancies has not been determined, it is noteworthy that significant tails were observed on these high mass peaks. Such tailing has previously been observed to arise from chemical adduction to or modification of the protein (Beavis & Chait, 1990a). Chemical adduction can arise, for example, by the noncovalent association of certain cations (e.g., Na^+, Cu^+) with the protein (Beavis et al., 1992). Chemical modification can arise, for example, by oxidation of the methionine residues, which renders the protein resistant to cleavage by CNBr or by modifications that may occur during electrophoresis (Chiari et al., 1992; Hall et al., 1993).

To estimate the amount of protein required to obtain an intense mass spectrum of the CNBr digestion products, the quantity of myoglobin applied to the SDS-polyacrylamide gel as well as the amount of protein recovered by electroblotting were determined by quantitative amino acid composition analysis. The amount of protein applied to the gel was determined to be 1.34 μg (79 pmol) and the amount recovered from the membrane 0.41 μg (24 pmol). The spectrum in Figure 2B was generated by applying 2.5% of the extracted sample (i.e., <0.6 pmol of each peptide) to the probe tip of the mass spectrometer.

Bovine synapsin I

Synapsins Ia and Ib are neuronal phosphoproteins whose properties are regulated by reversible protein phosphorylation. Synapsin Ia and Ib are closely related proteins containing 706 and 670 amino acids, respectively (Fig. 3), which co-purify under the conditions routinely employed. The isoforms are derived from alternative splicing of a single gene and share a common sequence from the amino-terminal to amino acid residue 661 (Sudhof et al., 1989). Six different protein kinases can phosphorylate synapsin I in vitro (Greengard et al., 1993). Previous work employing protein microsequencing techniques identified Ser-9 as the residue ("site 1") that is phosphorylated by either cAMP-dependent protein kinase or CaM kinase I and Ser-568 and Ser-605 as the residues ("sites 2 and 3") that are phosphorylated by CaM kinase II (Czernik et al., 1987; Sudhof et al., 1989). We undertook to determine by MALDI-MS the sites of phosphorylation of differentially phosphorylated bovine synapsins Ia and Ib separated by 2DE.

Initially, we determined the MALDI mass spectrum of a mixture of intact dephosphorylated synapsins Ia and Ib (calculated MWs = 74,519 Da and 70,441 Da, respectively). The experimental data (Fig. 4) yielded MWs = 74,650 ± 150 Da and 70,570 ± 150 Da, respectively, in agreement (within 0.2%) with the calculated MWs. The stoichiometric ratio of synapsin Ia to Ib in

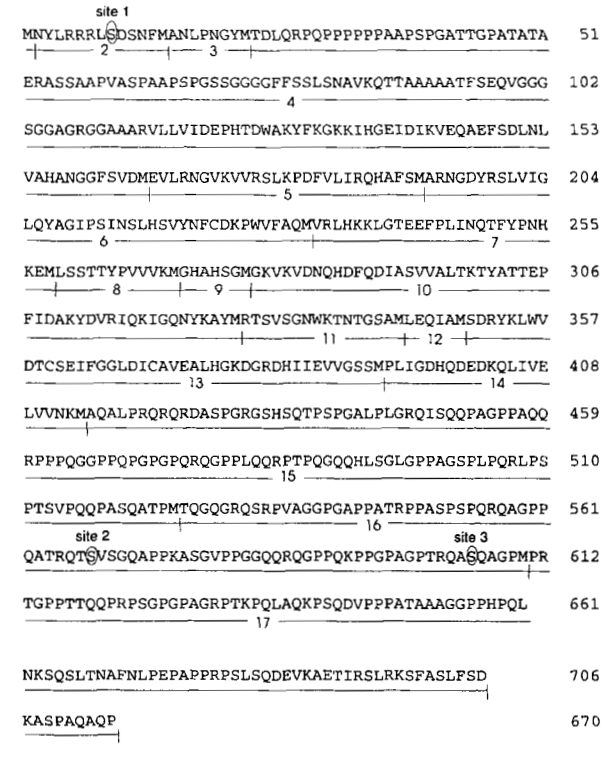

Fig. 3. Amino acid sequences of synapsin Ia and Ib. The CNBr cleavage sites are indicated together with the resulting fragments numbered from 1 to 17. The circled serines are the sites of phosphorylation (see text).

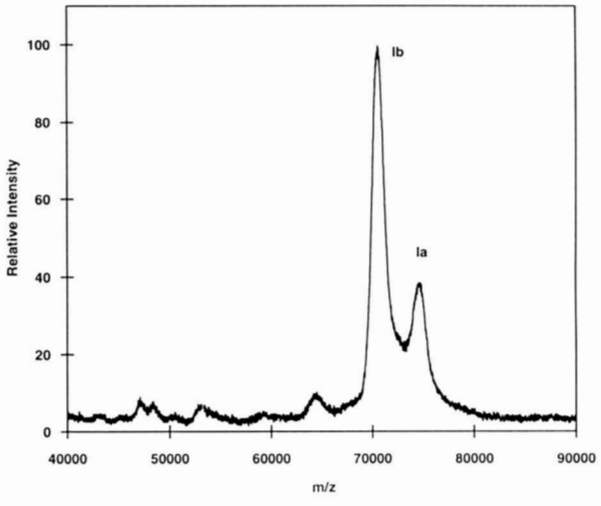

Fig. 4. MALDI mass spectrum of a mixture of dephosphorylated synapsins Ia and Ib.

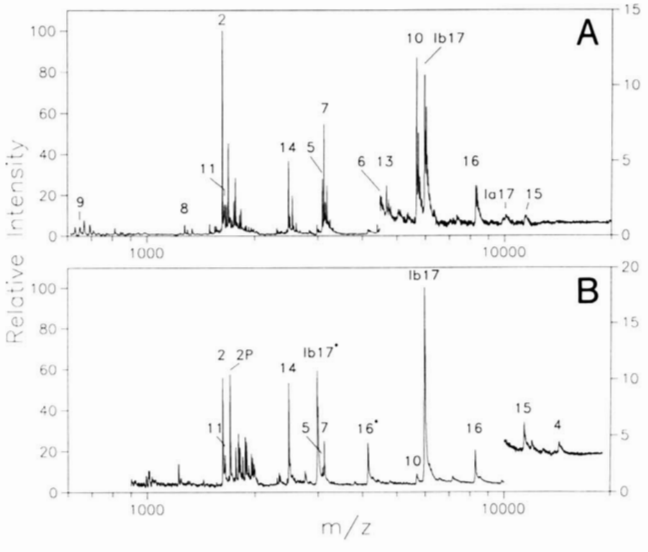

Fig. 5. MALDI mass spectrometric peptide maps generated by CNBr digestion of **(A)** synapsin Ia and Ib in solution and **(B)** synapsin Ib bound to an Immobilon CD membrane (center segment of the lower band shown in Fig. 6). To obtain spectrum A, 120 pmol of synapsins Ia and Ib was digested and <1% of the digest was subjected to MS analysis. To obtain spectrum B, 1–5 pmol of synapsin Ib was digested on the CD membrane and the total digest was subjected to MS analysis. The scale on the right-hand axis refers to the portion of the spectrum that has been expanded vertically. Asterisks designate peaks arising from doubly protonated peptides. The majority of the unlabeled peaks arise from the adduction of alkali metals and copper to the peptides.

the solution was estimated to be 1:2.2 by integration of the area under the mass spectral peak profiles. This ratio agrees well with the value obtained by quantitation of radioactive phosphate incorporation or by densitometric scanning of Coomassie blue-stained bands after SDS-PAGE using purified preparations of the protein (data not shown).

We next used MALDI-MS to investigate the peptide maps produced by CNBr digestion of (1) dephosphorylated synapsin I fragmented *in solution* and (2) synapsin I fragmented *on Immobilon CD* subsequent to 2DE separation of a mixture containing equimolar quantities of dephosphorylated synapsin I and synapsin I phosphorylated at site 1. Synapsins Ia and Ib each contain 16 methionine residues and therefore each yield 17 peptides upon complete cleavage with CNBr. The 16 amino-terminal peptides (designated 1, 2, 3, . . . , 16) from synapsin Ia are identical with those from synapsin Ib because they arise from the common amino-terminal sequence, whereas the remaining carboxyl-terminal peptides from Ia and Ib (designated as Ia17 and Ib17) are distinct. A MALDI-MS peptide map for a CNBr digest of unphosphorylated synapsins Ia and Ib carried out in solution is shown in Figure 5A. The proteins were cleaved with CNBr in HCl, dried under vacuum, and redissolved prior to application to the mass spectrometric probe in 30 mM 4-HCCA in 2.5% aqueous TFA:60% ACN (v/v). Seventeen out of the 18 CNBr fragments predicted from a mixture of unmodified synapsins Ia and Ib (fragments 1–16 plus Ia17 and Ib17) were detected, although signals for fragments 3, 4, and 12 were very weak. The single unobserved fragment was a homoserine lactone residue (mass = 101 Da) derived from the amino-terminal methionine residue. With the exception of the 2 largest fragments (4 and 15), the measured masses for the observed fragments (Table 1) were in close agreement with the calculated values. The MALDI-MS peptide map from synapsin Ib cleaved with CNBr on the Immobilon CD membrane after 2DE and isolation of the protein by electroblotting is shown in Figure 5B. Cleavage fragments were extracted with 30 mM 4-HCCA in 2.5% aqueous TFA:30% ACN (v/v). The CNBr cleavage was performed on the central segment of the lower band shown in Figure 6. The spectrum of fragments observed was less complete than that obtained from CNBr digestion of synapsin I in solution. Only 11 out of the expected 18 fragments from synapsin Ib (fragments 1–16, Ib17, and a fragment representing a phosphorylated peptide designated fragment 2P [see below]) were detected. Fragments 6, 8, and 13 were missing from the spectrum,

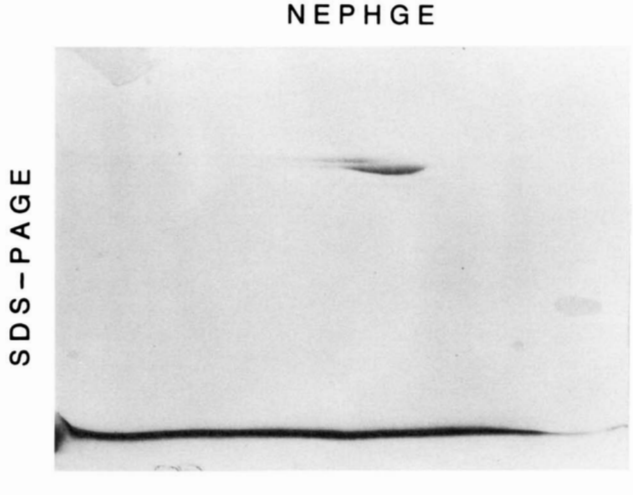

Fig. 6. Two-dimensional gel electrophoretic separation of synapsin mixture I by NEPHGE/SDS-PAGE 2DE (see text). A total of 3 μg of protein was applied to the gel. The separated proteins were stained with Coomassie blue. The more acidic portion of the gel is toward the left-hand side. The molecular weights of synapsin Ia and Ib, determined by MS, were 74,650 ± 150 Da and 70,570 ± 150 Da.

Table 1. *Comparison of the predicted and measured masses of fragments of synapsin I*[a]

CNBr fragment number	Calculated mass (Da)	Measured mass (Da) Digestion in solution	Δ	Digestion on membrane[b]	Δ
1	101.1	–	–	–	–
2	1,623.8	1,624.2	+0.4	1,624.7	+0.9
3	830.9	831.6	+0.7	–	–
4	14,029.3	13,968	−61	14,300	+271
5	3,091.7	3,092.2	+0.5	3,092.5	+0.8
6	4,484.1	4,483.3	−0.8	–	–
7	3,122.6	3,122.7	+0.1	3,122.8	+0.2
8	1,276.5	1,276.5 (c)	–	–	–
9	647.7	648.9	+1.2	–	–
10	5,675.4	5,675.6	+0.2	5,675.9[c]	+0.5
11	1,648.8	1,647.9	−0.9	1,648.8 (c)	–
12	655.8	655.5	−0.3	–	–
13	4,675.3	4,673.7	−1.6	–	–
14	2,485.9	2,486.0	+0.1	2,485.8	−0.1
15	11,362.6	11,387	+24	11,389	+26
16	8,277.1	8,277.2	+0.1	8,278	+0.9
Ia17	10,049.3	10,049	0	10,049	0
Ib17	5,970.7	5,970.7 (c)	–	5,970.7 (c)	–

[a] Fragments were produced by digestion with CNBr (1) in solution and (2) on the Immobilon CD blotting membrane. Δ denotes the deviation of the experimentally determined mass from the predicted mass; (c) denotes mass used as calibrant.

[b] The masses were determined for peptides obtained from the lower band shown in Figure 6 (synapsin Ib) with exception of the peptide Ia17, which was obtained from the top band of Figure 6 (synapsin Ia).

[c] The accurate mass determination for fragment 10 (when using 4-HCCA in 2.5% aqueous TFA:30% ACN [v/v] as the extraction solution) proved difficult to obtain because of its low peak intensity compared with the interfering peak arising from the doubly charged ion (m/z = 5,695 Da) of fragment 15. The listed value was obtained from a separate spectrum using 4-HCCA in 2.5% aqueous TFA:60% ACN (v/v) as the extraction solution.

while fragments 1, 3, 9, and 12 were in a lower mass region where the spectrum was not recorded due to the relatively high background. Again, with the exception of the largest fragments (4 and 15), the measured masses for the observed fragments (Table 1) were in close agreement with the calculated values.

As in the myoglobin study, the observed distribution of intensities of the various peptide fragment ion peaks in the mass spectra was found to be dependent on the composition of the extracting solution. This finding is illustrated in Table 2, which summarizes the observed peak intensities classified as strong (++), weak (+), or absent (−) as a function of 3 different extraction solutions. The use of a high concentration of organic solvent in the mixture (solutions B and C) promoted the extraction/solubilization of the larger and/or hydrophobic peptides. Solutions B and C were approximately equivalent in their ability to extract and dissolve the synapsin peptide fragments. Peptides that yielded either no mass spectrometric response or a very weak response were characterized by their large degree of hydrophobicity and/or the absence of readily ionizable Arg or Lys residues.

Table 2. *Comparison of the mass spectral intensities of peaks arising from the CNBr fragments of 2DE-separated synapsins using 3 different extraction solutions*[a]

CNBr fragment number	Calculated mass (Da)	A	B	C
1	101.1	–	–	–
2	1,623.8	++	++	++
3	830.9	–	–	–
4	14,029.3	+	+	+
5	3,091.7	+	++	++
6	4,484.1	–	–	–
7	3,122.6	++	++	++
8	1,276.5	–	–	–
9	647.7	–	–	–
10	5,675.4	+	++	++
11	1,648.8	++	+	+
12	655.8	–	–	–
13	4,675.3	–	+	+
14	2,485.9	++	++	++
15	11,362.6	+	+	+
16	8,277.1	++	++	++
Ia17	10,049.3	+	+	+
Ib17	5,970.7	++	++	++

[a] Extraction solutions: A, 30 mM 4-HCCA in 2.5% aqueous TFA: 30% ACN (v/v); B, 30 mM 4-HCCA in 2.5% aqueous TFA:60% ACN (v/v); C, 22 mM 4-HCCA in 1.9% aqueous TFA:45% ACN:25% HFIP (v/v/v). The observed intensities are classified as strong (++), weak (+), or absent (−).

Determination of peptide fragments containing sites of phosphorylation

We finally used MALDI-MS peptide mapping of synapsins separated by 2DE for the determination of sites of phosphorylation. Two different mixtures of phosphorylated and unphosphorylated synapsins were analyzed. The first (mixture I) consisted of unphosphorylated synapsins Ia and Ib combined in a 1:1 ratio with synapsins Ia and Ib phosphorylated in vitro at site 1 using cAMP-dependent protein kinase. The second sample (mixture II) consisted of unphosphorylated synapsin Ia and Ib combined in a 1:1 ratio with synapsins Ia and Ib phosphorylated in vitro at sites 2 and 3 with CaM kinase II. A total of 3 μg (12 pmol synapsin Ia, 28 pmol synapsin Ib) of each protein mixture was loaded onto a gel and subjected to 2DE employing nonequilibrium pH gel electrophoresis in the first dimension, followed by SDS-PAGE (NEPHGE/SDS-PAGE). Separated proteins were electroblotted onto Immobilon CD membranes and detected by reverse staining of the membrane. Figure 6 shows a representative result for protein mixture I. Whereas synapsins Ia (upper band) and Ib (lower band) were separated into 2 distinct bands in the molecular weight dimension, the different phosphorylation states were not well resolved in the NEPHGE dimension, due to the basic isoelectric point of these proteins. However, we anticipated that the more acidic, phosphorylated protein species would predominantly be present in the left-hand part of the band (with lower isoelectric point) and the nonphosphorylated species would be more prevalent in the right-hand (higher isoelectric point) region of the band. To assess whether such separation was occurring, each band on the blotting membrane was divided

into 3 approximately equal segments, which were individually cleaved and analyzed by MALDI-MS. The total amounts of protein present on each segment (assuming ~50% transfer efficiency to the membrane) were estimated to range between 1 and 5 pmol.

Analysis of synapsin mixture I separated by 2-dimensional electrophoresis

A sample of synapsin mixture I was prepared as described above (Fig. 6), and the 2 protein bands on the Immobilon CD membrane were each cut into 3 segments, which were individually subjected to CNBr digestion. Partial MALDI mass spectra from the recovered cleavage fragments encompassing the peaks corresponding to fragment 2 (containing phosphorylation site 1) and fragment 16 (containing phosphorylation sites 2 and 3) are shown in Figure 7. The experimentally determined MWs of fragments 2 and 16 were 1,624.7 Da and 8,278.0 Da, respectively. These values are in accord (within the experimental error) with the calculated values of 1,623.8 Da and 8,277.1 Da. A peak (designated 2P) representing a phosphorylated peptide was observed with an MW 80 Da higher than fragment 2. Although the mixture of unphosphorylated and phosphorylated proteins was not well resolved in the NEPHGE dimension (Fig. 6), the spectra obtained from different regions of the band confirmed the expected migration pattern of the differentially phosphorylated proteins. The sample derived from the more acidic segment of the top band (Fig. 7C) exhibited a considerably higher ratio of the phosphorylated peptide (2P) to the unphosphorylated peptide (2) than that obtained from the more basic segment (Fig. 7A). The spectrum obtained from the middle segment (Fig. 7B) showed an approximately equal distribution of the 2 protein species. Analogous results were obtained from the lower band (Fig. 7D,E,F). As predicted from the known consensus site requirements of cAMP-dependent protein kinase, no evidence of phosphorylation is observed on fragment 16, containing sites 2 and 3. In addition to demonstrating better resolution than 2DE alone, these results show that the mass spectrometric measurement of CNBr fragments provides a means for detecting the presence of the phosphorylated peptide in a peptide mixture and for the assignment of the site of phosphorylation to a specific peptide. In cases in which the phosphopeptide is known to contain a single phosphorylatable residue, the technique will be sufficient to determine the site of phosphorylation. When more than 1 phosphorylatable residue is present within a given peptide of known sequence, parallel analysis of phosphoamino acid content, together with a knowledge of the characteristic consensus site requirements for a large number of protein kinases (Kemp & Pearson, 1990), can also assist in establishing the exact site of phosphorylation. Alternatively, the sites of serine/threonine phosphorylation and tyrosine phosphorylation can be determined by chemical sequencing of the isolated phosphopeptide (Aebersold et al., 1991; Meyer et al., 1991; Wettenhall et al., 1991) or by tandem mass spectrometric analysis (Biemann, 1992).

Analysis of synapsin mixture II separated by 2-dimensional electrophoresis

Synapsin mixture II was subjected to an analysis similar to that described above. While peptide 2P was not observed in these samples, peptide 16 (which contained phosphorylation sites 2 and 3) underwent phosphorylation, as evidenced by the presence of peaks with MWs 80 Da and 160 Da greater than that of unmodified peptide 16 (data not shown). We undertook to isolate the 2 serine residues (i.e., the 2 potential sites of phosphorylation) by subfragmentation of phosphorylated peptide 16 into 2 products, each containing 1 site of phosphorylation. Synapsin Ia and Ib bands on the Immobilon CD membrane were each cut into 3 segments representing the acidic, central, and basic sections and subjected to sequential Lys-C and CNBr digestions prior to mass spectrometric analysis of the resulting fragments. Lys-C digestion was expected to cleave CNBr fragment 16 between phosphorylation sites 2 and 3, yielding peptides a (residues 577–610, containing site 3) and b (residues 526–576, containing site 2). Partial mass spectra of the regions encom-

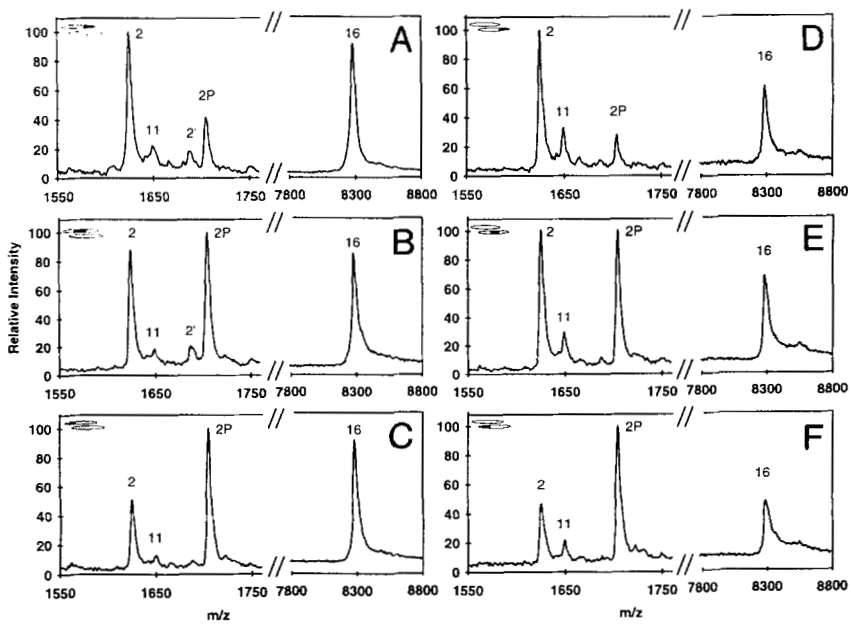

Fig. 7. Detailed portions of the matrix-assisted laser desorption mass spectra of CNBr digests of synapsin mixture I (see text) after separation by 2DE and electroblotting. **A–C:** Synapsin Ia. **D–F:** Synapsin Ib. Peaks corresponding to fragments 2 and 16 (see Fig. 3) are shown. The portion of the blot derived from the 2DE separation that was examined in each spectrum is indicated in the small inset in the upper left corner of each panel. The peaks labeled 2′ arise through adventitious adduction of copper to peptide 2.

passing peptides a and b derived from the acidic, central, and basic segments of synapsins Ia and Ib are shown in Figure 8. The experimentally determined molecular weights of peptides a and b were 3,242.4 Da and 5,052.9 Da, respectively. These values are in accord (within the experimental error) with the calculated values of 3,242.6 Da and 5,052.6 Da. As was the case with mixture I, the mass spectrometric analysis provided both direct detection of the states of phosphorylation and assignment of the phosphate ester groups to a specific segment of synapsins Ia and Ib as well as better resolution as compared to 2DE of the proteins containing different amounts of phosphorylation.

Conclusions

The technique described here provides a rapid, sensitive means for analyzing putative posttranslational modifications of proteins that have been separated by 2DE. We predict that this technique will have quite general utility because most proteins can be separated by 2DE and caused to undergo hydrolysis, which can be informative. The electroblotting approach appears well suited for MALDI-MS because it allows ready removal of SDS, an important requisite for obtaining high-quality mass spectra (Beavis & Chait, 1990b). As demonstrated for synapsin I, the present approach can assist in the determination of the sites of phosphorylation in proteins and, more generally, can be used for the determination of the molecular basis of differential migration of protein isoforms in 2D gels. Other posttranslational modifications should also be readily measurable provided that the modifications are stable to the chemical or enzymatic treatment used to produce the peptide map. Present limitations of the approach include the difficulty of obtaining complete peptide maps from a given protein and the compromised mass accuracy achieved for the larger peptide fragments. In particular, small peptides lacking basic residues may give relatively weak MS responses. Current efforts are focused on further improving the efficiency of peptide extraction from the electroblotting membrane and further reducing discrimination effects through improved methods for producing the matrix/peptide sample. Finally, as previously demonstrated (Henzel et al., 1993; James et al., 1993; Mann et al., 1993; Pappin et al., 1993; Yates et al., 1993), the technique is also well suited for the facile identification of proteins by comparison of the measured masses determined from the peptide maps with appropriate protein sequence databases. The relative merits of using CNBr peptide maps for this purpose compared with the previously employed tryptic peptide maps remain to be determined.

Materials and methods

Materials and instrumentation

Two-dimensional gel electrophoresis was performed using an Investigator 2DE System (Millipore, Bedford, Massachusetts) or a Bio-Rad Mini Protean II electrophoresis system (Bio-Rad, Richmond, California). Electroblotting was performed in a Mini Protean II electroblotting system (Bio-Rad). Mass spectrometry of peptides in this study was carried out on a MALDI time-of-flight MS constructed at The Rockefeller University and described elsewhere (Beavis & Chait, 1989, 1990a). Samples were concentrated in a Speedvac vacuum concentrator (Savant Instruments, Inc., Farmingdale, New York). Amino acid analysis was carried out on a model 420 amino acid analyzer (Applied Biosystems, Foster City, California) using standard precolumn PITC derivatization protocols.

All chemicals and reagents used for electrophoresis, including carrier ampholytes (nominal pH range 3–10), and Immobilon CD stain were from Millipore; Immobilon and Immobilon CD electroblotting membrane were also purchased from Millipore. Chemicals and reagents included α-cyano-4-hydroxycinnamic acid as the laser desorption matrix, CNBr, and HFIP (Aldrich, Milwaukee, Wisconsin); endoproteinase Lys-C (sequencing grade, Boehringer-Mannheim, Indianapolis, Indiana); and horse heart myoglobin (Sigma, St. Louis, Missouri). Synapsin I was purified from calf brains obtained from a local slaughterhouse,

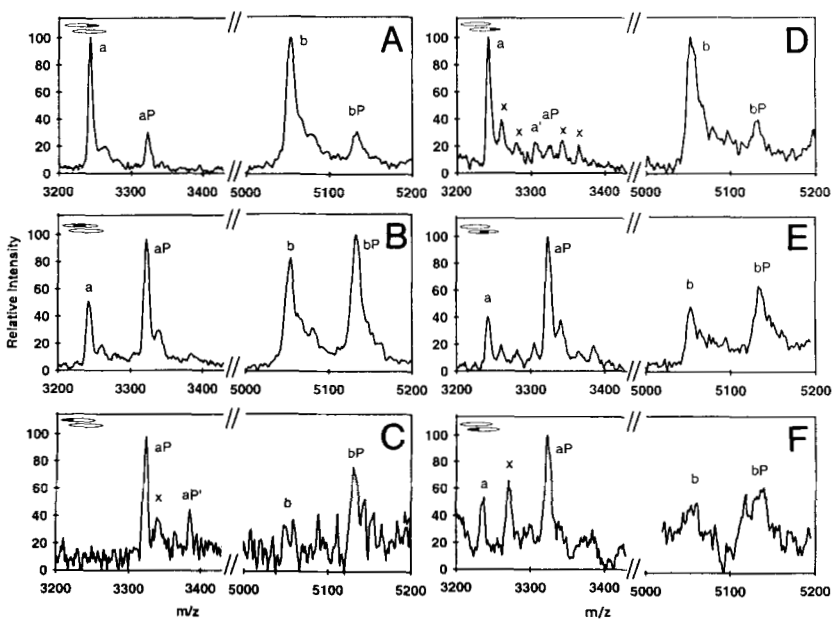

Fig. 8. Detailed portions of the matrix-assisted laser desorption mass spectra of sequential Lys-C and CNBr digests of synapsin mixture II (see text) after separation by 2DE and electroblotting. **A–C:** Synapsin Ia. **D–F:** Synapsin Ib. Peaks corresponding to fragments a (residues 577–610, containing site 3) and b (residues 526–576, containing site 2) are shown. The portion of the blot derived from the 2DE separation that was examined in each spectrum is indicated in the small inset in the upper left corner of each panel. The peaks labeled X arise from unidentified impurities. The peaks labeled a′ and aP′ arise through adventitious adduction of copper to peptides a and aP, respectively.

as described by Bahler and Greengard (1987). Samples of synapsin I were phosphorylated with the catalytic subunit of cAMP-dependent protein kinase (kindly provided by Angus Nairn and Atsuko Horiuchi, The Rockefeller University) or with CaM kinase II as described by Czernik et al. (1987).

Two-dimensional gel electrophoresis

Because of their highly basic isoelectric point (>10.5; Valtorta et al., 1992), it proved necessary to separate the synapsin isoforms (containing the various phosphorylation states of synapsin Ia and Ib) by nonequilibrium pH gel electrophoresis (O'Farrell, 1975; O'Farrell et al., 1977). The synapsins were dissolved at a concentration of 0.6 mg/mL in 9 M urea, 4% NP-40, 2.2% ampholytes, and 100 mM DTT. Three-microgram aliquots of protein were loaded on each gel. A first dimension focusing gel (18 cm long) was cast in a 26-cm glass tube with a 1-mm inner diameter. This gel had 4.11% total acrylamide concentration and contained 0.35% crosslinker and 9.5 M urea, 2% NP-40, and 5 mM CHAPS. Synapsin samples were loaded on the acidic side of the gel and prefocused at a constant current of 110 mA for 1 h, at which time the voltage was set at 1,000 V and maintained at that level for 4 h. After NEPHGE, a 6-cm-long portion of the gel containing the basic proteins was equilibrated in 375 mM tris/HCl (pH 8.8), 3% SDS, 50 mM DTT, and 0.01% bromophenol blue for 2 min and placed on top of a 10.26% total acrylamide concentration/0.87% crosslinker mini-gel. Mini-gels were run at 100 V constant voltage for 1.5 h.

Protein electroblotting

Proteins separated by 2DE were transferred to an Immobilon CD membrane by wet electroblotting, following the conditions described by Patterson et al. (1992) and Hess et al. (1993). The transfer buffer contained 25 mM tris, 192 mM glycine, and 10% methanol. Two gels were transferred per tank for 1.5 h at 50 V (constant voltage). Following transfer, proteins were located on the CD membrane by negative staining using the Immobilon CD stain following the procedure supplied by the manufacturer. Protein-containing areas were excised, placed wet in plastic tubes, and stored for analysis at −20 °C.

Sample preparation prior to chemical and enzymatic cleavage

The quality of the mass spectra of peptides and proteins obtained by MALDI-MS is adversely effected by the presence of SDS (Beavis & Chait, 1990b) and stains (unpublished findings from the authors' [W.Z. and B.T.C.] laboratory). It is therefore important to remove, as completely as possible, residual SDS that may be present on the membrane as well as the bulk of the staining compound. A washing procedure was devised to selectively remove SDS and stain from the membrane without incurring loss of the protein. Prior to washing, the protein spot was identified through its lighter color on the reverse-stained membrane and the edge of the spot was marked with pinholes. A piece of membrane somewhat larger than the marked region was cut out and washed for 5 min with intermittent agitation in a few hundred microliters of pure methanol. The wash solution was discarded and the procedure was repeated 2 more times. The protein spot was then cut out along the edge marked with the pinholes before being further divided into approximately 1-mm^2 pieces. These small pieces of membrane allowed the use of small working volumes of solution during protein cleavage and subsequent peptide extraction. In the case of synapsin I, each protein spot was cut out and further subdivided into 3 segments representing the acidic, central, and basic regions of the spot, respectively. Mass spectrometric analysis of proteins on these individual segments enabled the resolution of differentially phosphorylated species that were not resolved by 2DE.

Chemical and enzymatic cleavage of proteins

Proteins were cleaved into peptide fragments directly on pieces of membrane with CNBr and/or Lys-C. All chemical reactions on the membrane-bound proteins and subsequent extraction of the resultant peptides were carried out in 0.5-mL-capacity polypropylene microfuge vials.

CNBr

Proteins were digested by adding to the appropriate pieces of washed membrane a small volume (10 μL) of a solution of 0.5 M CNBr in 0.1 N HCl. The reaction was carried out at 25 °C for 1 h under dry nitrogen in the dark. After reaction, the solution was withdrawn and the membrane pieces allowed to dry in an open vial. Attempts to dry the membrane pieces by vacuum evaporation in a Speedvac were abandoned because vacuum drying was found to cause irreversible loss of the peptides with respect to extraction from the membrane. In a preliminary study of the CNBr digestion of myoglobin under the conditions described above, an overwhelming majority of the peptide products was observed to remain bound to the membrane subsequent to reaction. Thus, the digestion solution was discarded.

Endoproteinase Lys-C

To obtain additional sites of cleavage, Lys-C was used in combination with CNBr. The Lys-C digestion was carried out prior to CNBr digestion under 2 different sets of conditions. The first set employed a rapid cleavage using a high enzyme:substrate ratio (1:1 [w/w]). The membrane pieces were incubated with enzyme solution (0.1 g/L Lys-C, 50 mM Tricin, 10 mM EDTA, pH 8.0) for 5 min at 37 °C. The second set of conditions involved a longer incubation (24 h) using an enzyme:substrate ratio of 1:100 (w/w). In comparison to the first set of conditions, the second set yielded spectra with a considerably lower abundance of background peaks that likely arise from autolysis of Lys-C. After reaction, the digestion solution was withdrawn and discarded and the membrane pieces were rinsed with 10 μL of 50 mM NH_4HCO_3 (pH 8.5) to remove residual enzyme. The NH_4HCO_3 wash was discarded. Although there were indications in a study of myoglobin that a small fraction of the peptide fragments was extracted into the enzyme solution, the bulk of all of the peptide fragments remained attached to the membrane.

Peptide extraction from the membrane

Efficient extraction of peptides from the blotting membrane is a prerequisite for the high-sensitivity MALDI-MS analysis

of proteins separated by gel electrophoresis. The Immobilon CD membrane has a cationic surface so that polypeptides are retained predominantly by ionic interactions, which can be disrupted at acidic pH. Even though interactions between polypeptides and the membrane may be easily broken by acidic solvents, efficient extraction can be difficult to achieve because the peptides generated by CNBr and enzyme digestion are often highly insoluble in aqueous buffers.

Solutions used for peptide extraction consisted of mixtures of acidified water and organic solvent in various ratios. In addition, a 30 mM concentration of the laser desorption matrix 4-HCCA was added to the extraction solution to facilitate direct mass spectrometric examination of the extracted peptides. Because of the high binding affinity of this matrix for peptides and proteins, the cinnamic acid derivative may assist in the extraction process. A solution containing 30 mM 4-HCCA in 2.5% TFA:30% ACN (v/v) was found to extract many of the peptide fragments generated in the present study. Extraction solutions containing larger proportions of organic solvent were found to more efficiently extract the more hydrophobic peptide fragments. Two such solvent mixtures (among many others) that were found to be useful are: (1) 30 mM 4-HCCA in 2.5% TFA: 60% ACN (v/v) and (2) 15 mM 4-HCCA in 1.25% TFA:15% ACN:50% HFIP (v/v/v). The extraction solution was added to the membrane pieces to yield estimated peptide concentrations of 1–2 μM. When there was estimated to be less than 2 pmol of protein on the membrane, only 1 μL of extraction solution was added. In all cases, the membrane pieces were subjected to bath sonication for 30 s to promote extraction.

Quantitation of blotted proteins

Membrane-bound proteins and proteins in solution were quantitated by amino acid composition analysis. Protein amounts electroblotted onto Immobilon CD were determined by preparing identical samples in a parallel experiment electroblotted onto an Immobilon P membrane, assuming equal transfer efficiency for both membranes. Peptide bond hydrolysis and quantitative analysis were performed using standard protocols.

MALDI-MS

The peptide mixtures generated by digestion on the membrane were subjected to analysis by MALDI-MS (Beavis & Chait, 1990b; Hillenkamp et al., 1991). The mass spectra were acquired by adding together the individual spectra from 200 laser shots. Samples were prepared for laser desorption analysis by placing 0.5–1.0 μL of the peptide extraction solution, containing 4-HCCA (see above) (Beavis et al., 1992), on the mass spectrometer probe tip, followed by air drying at room temperature. The sample was then inserted into the mass spectrometer and analyzed. Horse heart myoglobin was used as the primary calibrant of the mass spectra. Secondary (more accurate) calibrations were performed using selected proteolytic degradation products (as indicated in Table 1). A structureless background is sometimes observed to underlie the sharp spectral peaks in MALDI-MS. When present, these smooth backgrounds were subtracted from the mass spectra to accentuate the sharp peaks. Masses were assigned to the peaks in the spectrum by computer-controlled determination of the peak centroids.

Note added in proof

In a personal communication (to B.T.C.), Scott Patterson (Amgen, Thousand Oaks, California) reports a significant release of peptides from certain membrane-bound proteins by Lys-C into the digestion buffer.

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