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Analytical and micropreparative peptide mapping by high performance liquid chromatography/electrospray mass spectrometry of proteins purified by gel electrophoresis

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Abstract

We report the use of microbore reverse-phase high performance liquid chromatography connected on-line to an electrospray mass spectrometer for the separation/detection of peptides derived by proteolytic digestion of proteins separated by polyacrylamide gel electrophoresis. A small fraction (typically 10% of the total) of the peptides eluting from the column was diverted through a flow-splitting device into the ion source of the mass spectrometer, whereas the majority of the peptide samples was collected for further analyses. We demonstrate the feasibility of obtaining reproducible peptide maps from submicrogram amounts of protein applied to the gel and good correlation of the signal detected by the mass spectrometer with peptide detection by UV absorbance. Furthermore, independently verifiable peptide masses were determined from subpicomole amounts of peptides directed into the mass spectrometer.

The method was used to analyze the 265-kDa and the 280-kDa isoforms of the enzyme acetyl-CoA carboxylase isolated from rat liver. The results provide compelling evidence that the two enzyme isoforms are translation products of different genes and suggest that these approaches may be of general utility in the definitive comparison of protein isoforms. We furthermore illustrate that knowledge of peptide masses as determined by this technique provides a major advantage for error-free data interpretation in chemical high-sensitivity peptide sequence analysis.

Keywords: acetyl-CoA carboxylase; mass spectrometry; peptide mapping; polyacrylamide gel electrophoresis; reverse-phase high performance liquid chromatography

The characterization of proteins separated by one- or two-dimensional polyacrylamide gel electrophoresis on the primary structural level is a technique of increasing importance in protein biochemistry and cell biology. Since gel electrophoresis is the method with the highest resolving power for the separation of proteins, extremely complex protein mixtures can be resolved in a single operation (O'Farrell, 1975). Gel electrophoresis is furthermore compatible with resolving very small amounts of proteins, and differentially modified (e.g., differentially phosphorylated) forms of the same polypeptide can be separated and subsequently recognized as such by immunoblotting (Towbin et al., 1979). Finally, the introduction of software for quantitative subtractive analysis of two-dimensional gel patterns is used for the simultaneous identification of sets of proteins, the expression of which is co-regulated in particular physiological or pathological conditions (reviewed by Aebersold & Leavitt [1990]). Extended charac-

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Abbreviations: ACC, acetyl-CoA carboxylase; CA, bovine carbonic anhydrase; CF-FAB, continuous-flow fast atom bombardment; electrospray, pneumatically assisted electrospray; i.d., inner diameter; MS, mass spectrometer (spectrometry); RP-HPLC, reverse-phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TIC, total ion current.

terization of such proteins generally requires analysis on the primary structural level.

The most general relevant techniques include chemical or enzymatic fragmentation of isolated polypeptides in the gel matrix following electrophoresis (Eckerskorn & Lottspeich, 1989; Vanfleteren et al., 1992) or, after electrotransfer onto a suitable matrix, fragmentation on the electroblotting support (Aebersold et al., 1987; Iwamatsu, 1992; Patterson et al., 1992). In most cases, cleavage fragments are separated by reverse-phase high performance liquid chromatography, detected by UV absorbance of the peptide bond, and collected for further analyses such as peptide sequencing. Although detection of eluting peptides by UV absorbance is relatively sensitive and general, it does not yield information on the molecular nature of separated peptides or the degree of homogeneity of a peptide peak, parameters that are essential for successful data interpretation in chemical sequence analysis. In special cases, co-eluting peptides containing different ratios or compositions of amino acids with aromatic side chains can be distinguished by the use of photo diode array detectors (Grego et al., 1986), but this method is not general and is of limited value in high-sensitivity peptide mapping experiments.

Some of the most significant recent advances in the technology of mass spectrometry, for the characterization of biological molecules, have been achieved in the development and application of new ionization techniques (reviewed by Carr et al. [1991]). For the analysis of biological polymers, matrix-assisted laser desorption (Karas & Hillenkamp, 1988), and electrospray/ion spray (pneumatically assisted electrospray) (Covey et al., 1988; Fenn et al., 1989) have emerged as the most useful tools. Pneumatically assisted electrospray (electrospray) ionization is ideally suited for combining HPLC with MS detection (Whitehouse et al., 1985; Bruins et al., 1987; Griffin et al., 1991). The ease with which HPLC can be coupled on-line to the MS utilizing electrospray ionization has led to a rapid expansion of the analytical applications of the technique, with particular importance in peptide mapping for protein sequencing (Hemling et al., 1990; Covey et al., 1991; Griffin et al., 1991; Huang & Henion, 1991; Ling et al., 1991; Hunt et al., 1992).

Here we demonstrate the use of an electrospray MS connected on-line with a microbore RP-HPLC system as a detector for analytical and micropreparative peptide mapping of low-picomole amounts of proteins separated by gel electrophoresis. By flow splitting of the column eluate, a fraction (typically 10%) of the sample was diverted into the ion source of the MS while the majority of the sample was collected for sequence analysis. We show that in this configuration the masses of peptide fragments were determined when as little as 17 pmol of a protein was applied to a sodium dodecyl sulfate-polyacrylamide gel, and that peptide detection by electrospray MS correlated well with detection by UV absorbance.

We have used this technique to precisely determine the masses of a large number of peptides recovered from the 265-kDa and 280-kDa isoforms of the enzyme acetyl-CoA carboxylase isolated from rat liver. The results demonstrate differences between the isoforms of ACC that are so substantial that the polypeptides are most likely translation products of different genes.

The method described here is also ideally suited for the identification of peptides containing modified amino acid residues, and yields peptide masses of numerous cleavage fragments of a single polypeptide. These data can be used for identifying proteins by screening suitably formatted protein sequence databases (Henzel et al., 1989, 1993; Yates et al., 1991; Pappin et al., 1993). Furthermore, knowledge of peptide masses and purity of the peptide sample are essential for error-free data interpretation in chemical high-sensitivity peptide sequence analysis.

Because the technique described here addresses many common analytical problems in protein biochemistry, it is anticipated that it will find widespread use for the characterization, at the primary structural level, of proteins separated by one- and two-dimensional gel electrophoresis.

Results and discussion

Correlation of peptide detection by UV absorbance and mass spectrometry

Relative sensitivities

To evaluate the relative sensitivities of peptide detection by UV absorbance of the peptide bond and by electrospray MS, sequential UV absorbance and MS detection was done during peptide mapping of proteins, utilizing the instrument configuration illustrated (Fig. 1). A sample of bovine carbonic anhydrase (350 pmol) was subjected to SDS-PAGE, electroblotted onto Immobilon CD, and cleaved on the matrix with trypsin. Resulting fragments were released into the supernatant and separated by microbore RP-HPLC. Established protocols for the sample preparation were followed (Patterson et al., 1992). In general, peptide profiles detected by UV absorbance or by MS total ion current responses (Fig. 2A,B) were qualitatively similar, although there were some notable exceptions. Thus, although the relative sizes of peptide peaks were mostly comparable by the two techniques, peptides T1 and T2 showed a greater relative UV absorbance. These peptides contain the aromatic residue tryptophan within a short peptide sequence (Fig. 3, sequence alignment of CA; Sciaky et al., 1976), resulting in higher extinction coefficients and consequently greater response factors by UV detection. Precise, quantitative assessments of relative response factors of individual peptides were not possible from these data because the absolute amounts of each peptide eluted from the column were unknown

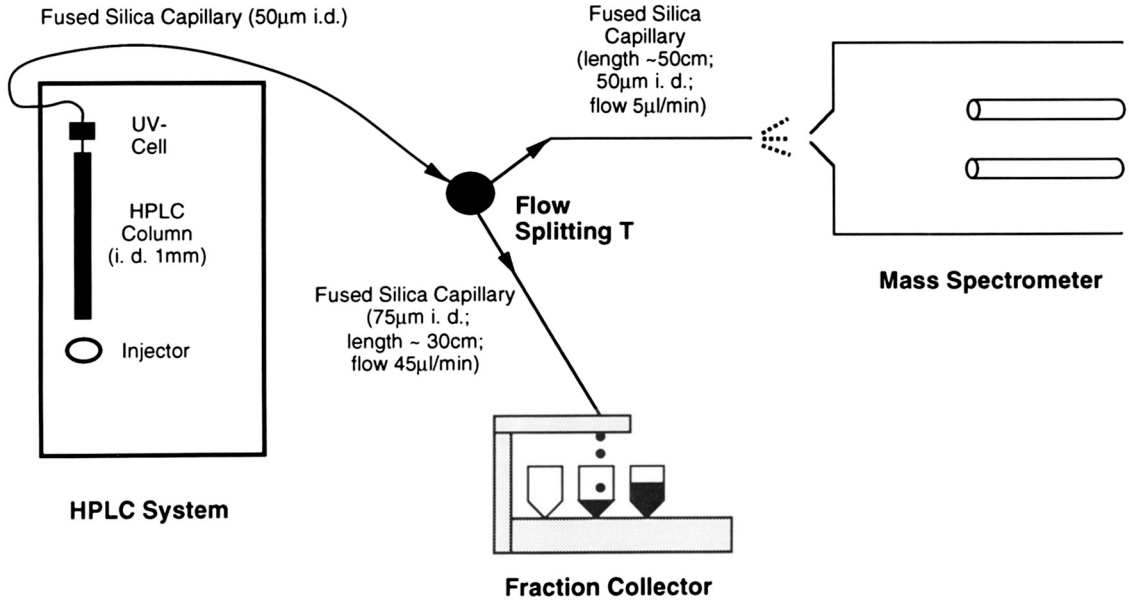

Fig. 1. Schematic diagram **of** microbore RP-HPLC/MS system.

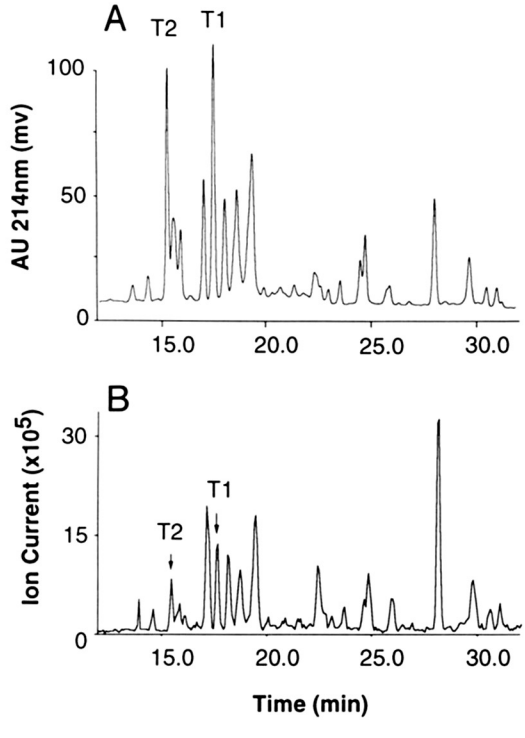

Fig. 2. Tryptic peptides from carbonic anhydrase separated by RP-HPLC and detected by **UV** absorption or by mass spectrometry. Carbonic anhydrase (350 pmol) was subjected to SDS-PAGE, electroblotted **on** Immobilon CD, and digested with trypsin **on** the membrane. Resulting peptides were separated **on** an RP-HPLC Reliasil C18 (I × 150mm, 300 A) column, using a TFA/acetonitrile solvent system as described in Materials and methods. **A:** UV chromatogram at 214 nm of the total digest. **B:** Total ion current of all masses between 400 and 2,400 Da as a function of elution time. Ten percent of the column effluent was diverted to the **MS** using the setup outlined in Figure I. TI and T2 mark tryptophan-containing tryptic peptides.

due to variability in digestion efficiency and recovery from the membrane and RP-HPLC column. From the data shown (Fig. **2A,B)** and our experience with quantitatively well-characterized peptide samples, it appears, however, that detection of tryptic peptides by **MS,** in contrast to detection by UV absorbance, is less dependent of the amino acid composition of the peptide.

Absolute sensitivity and optimal system configuration

It was our goal to design a system optimized for maximum **MS** sensitivity in conjunction with the recovery of the greatest percentage of the sample injected (Fig. 1). **A** low dead volume postcolumn flow splitter was implemented to recover 90% of the sample eluted from a 1-mm-i.d. microbore column. **A** comparison of the UV absorbance and TIC signals (Fig. **2A,B)** indicates a roughly equivalent signal to noise ratio, even though only 10% of the peptide samples were diverted into the **MS.** This could lead to the erroneous conclusion that the **MS** was approximately 10 times more sensitive than the UV detector. However, when the column effluent was not split and 100% of the sample entered the **MS,** the resulting signal was only **20%** greater than if 90% were diverted away from the **MS** for collection (data not shown). It was therefore the concentration of the sample in the eluant entering the detector that determined the response and ***not*** the absolute quantity of material. Thus, the behavior of the **MS** was that of a concentration-sensitive detector (Covey et al., 1991), similar to that observed with classical concentration-sensitive detectors. **As** a practical consequence of this observation, a large proportion of the

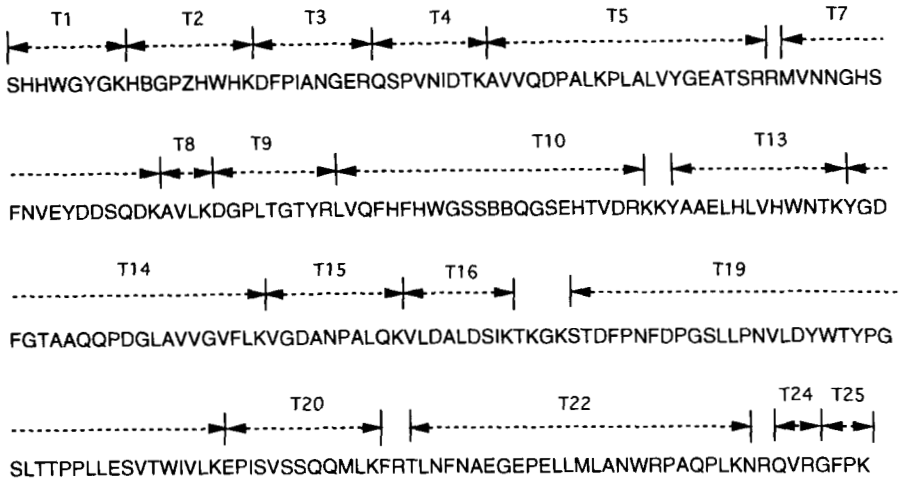

Fig. 3. Sequence of carbonic anhydrase (Sciaky et al., 1976). T1–T25 represent predicted tryptic peptides. B, Asp or Asn; Z, Glu or Gln.

sample could be recovered by postcolumn splitting without serious losses in sensitivity.

A corollary to this observation is the inverse square relationship between column diameter and absolute sensitivity. Provided that the chromatographic efficiency (peak widths) is preserved, fourfold increases in sensitivity can be achieved by decreasing the column diameter by a factor of 2. Packed capillary HPLC columns, ranging from 500 to 75 μm i.d. have provided significantly greater absolute sensitivities than 1-mm columns with MS detection (Huang & Henion, 1991; Hunt et al., 1992). However, the advantage of simultaneous molecular weight determination and preparative sample collection for further analysis is lost with packed capillaries for three reasons. First, although feasibility has been demonstrated (Hunt et al., 1992), it is experimentally difficult to stream split flows under 10 μL/min. At flow rates typically used with 75–320-μm-i.d. capillaries (low to sub-μL/min), stream splitting represents a difficult task. Second, chromatographic performance is often compromised in capillary columns as compared to wide-bore or microbore columns. Third, electrospray systems require minimum flow rates on the order of 2–5 μL/min to the MS to maintain a stable signal. With capillary columns that operate in the same range of flow rates, virtually no sample can be recovered for alternative analyses. The 1-mm column system was therefore chosen for these experiments as an optimal compromise between sample recovery and sensitivity of detection. High split ratios could be practically achieved (45 μL/min flow to collecting line, 5 μL/min to MS) while picomole-level sensitivity was maintained. The use of packed capillary columns of 500 μm i.d. operated at a flow rate of 12–15 μL/min provided enhanced sensitivity for these experiments. However, the low flows seriously challenged both the gradient delivery capabilities of present-day commercial HPLC pumping systems, which require the use of pre-injector splitters, and the ability to reliably collect samples.

Chromatographic fidelity

Introduction of the splitter arrangement and the electrospray interface could potentially introduce chromatographic band broadening and loss of resolution. In order to maintain the resolution of the RP-HPLC separation between the UV cell and the MS, it was essential to use low-diameter fused silica capillary tubing (50 μm) and short connections. The configuration shown in Figure 1 resulted in a delay between UV cell and electrospray of approximately 15 s, and the delay between UV cell and fraction collector was 5 s. In addition, comparison of the UV absorbance and TIC signals (Fig. 2A,B) shows that chromatographic efficiency has not been detectably compromised.

Sensitivity of LC/MS for peptide mapping of gel-separated proteins

In peptide mapping experiments, very high sensitivities of detection can only be fully exploited if the whole process including protein separation, fragmentation, and peptide recovery is adapted to the same microscale. We therefore attempted to assess the sensitivity of the entire analytical process most commonly utilized for protein structural characterization in combination with split-flow microbore LC/MS. Recently, we and others have developed high-sensitivity procedures for the preparation of peptide fragments from gel-separated proteins (Aebersold et al., 1987; Eckerskorn & Lottspeich, 1989; Iwamatsu, 1992; Patterson et al., 1992; Vanfleteren et al., 1992).

Aliquots of CA, quantitated by amino acid composition analysis, were separated by SDS-PAGE and electroblotted onto Immobilon CD membrane. Bands were detected by negative staining using the dye Quick-Stain, and individual protein bands were excised and digested with trypsin. The resulting peptide mixture, expected to contain fragments termed T1–T25 (Fig. 3), was analyzed using the system described in Figure 1 with the 90% split for sample recovery. Figure 4 displays the results from ex-

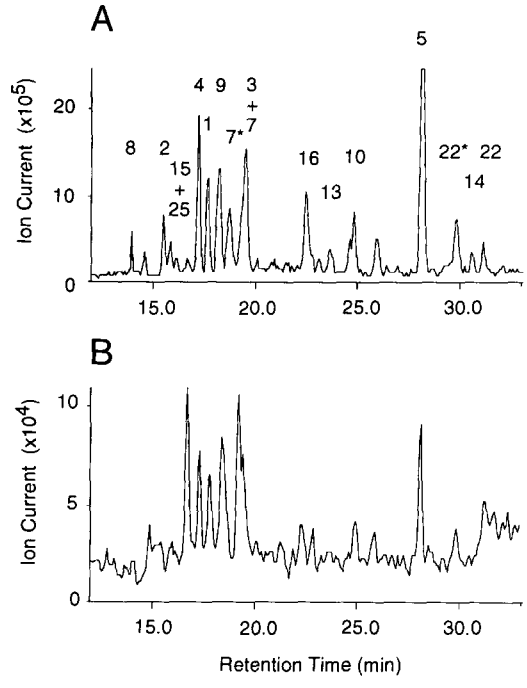

Fig. 4. Electrospray mass spectra of peptides from two different amounts of carbonic anhydrase. Samples (**A:** 350 pmol; **B:** 35 pmol) of the protein were subjected to SDS-PAGE, electroblotting, and tryptic fragmentation. Resulting peptides were separated by RP-HPLC prior to MS. The peptides numbered in A correspond to the peptides expected from tryptic digest of carbonic anhydrase with the published sequence (see Fig. 3).

periments in which aliquots of 350 pmol (Fig. 4A) and 35 pmol (Fig. 4B) of CA were applied to the separating gel. Peptides contained in individual peaks were identified by their molecular weight and by chemical sequence analysis of the collected fractions as designated (Fig. 3). All the detected peaks contained peptides that could be assigned to the primary structure of CA based on the determined molecular weights (±1 Da), with the exception of two peptides containing methionine residues. The two methionine-containing peptides (T7 and T22) were partially modified, resulting in earlier-eluting species with an additional mass of 16–17 Da, a mass difference compatible with oxidation. Because we observed the same degree of modification in solution-phase digests of CA that had not been subjected to SDS-PAGE, we concluded that gel electrophoresis, electroblotting, and digestion on the Immobilon CD membrane did not introduce peptide modifications detectable by MS. Although we have not specifically studied the N-terminal peptide of a protein following SDS-PAGE, we have repeatedly detected unmodified peptides containing lysine residues. From the chemical similarity of the α-amino group and the ε-amino group of lysine side chains, we deduce that the N-terminal of proteins does not become modified to a significant degree during the SDS-PAGE/electroblotting procedure. This result supports the experience that proteins generally do not become blocked for chemical N-terminal sequence analysis during gel electrophoresis, provided gel chemicals of a high degree of purity are used.

With the exception of T19, T20, and T24, all the expected tryptic peptides from CA within the scanned mass range were identified (Table 1). The mass determined for T1 was compatible with N-terminal acetylation of CA, as reported before (Filippi-Foveau et al., 1976). Partial sequence analysis of peptide T1 by tandem MS further confirmed T1 as the N-terminal, acetylated peptide (data not shown). The tripeptide T24 most likely did not bind to the RP-HPLC column. Because T19 and T20 were detectable neither by UV absorbance nor by MS and because these peptides were recovered at very low yields even if CA was digested in solution, we concluded that T19 and T20 were lost due to low solubility and/or irreversible absorption of the peptides. This interpretation is consistent with the experience of Filippi-Foveau et al. (1976), who reported poor solubility of peptides, including T19 and T20, un-

Table 1. *Masses of tryptic fragments of bovine carbonic anhydrase observed by HPLC-MS compared with calculated masses*[a]

Peptide no.	Theoretical MH^+ (avg.) (Da)	Observed MH^+ (Da)[b]
11	147.2	n.s.
12	147.2	n.s.
6	175	n.s.
18	204.3	n.s.
17	248.3	n.s.
23	289.3	n.s.
21	322.4	322.2
24	402.5	n.d.
8	430.6	430.2
25	448.5	448.2
16	974.1	973.2
9	980.0	979.0
4	1,002.1	1,002.7
15	1,013.1	1,013.6
1	1,014.1	1,013.6
3	1,019.1	1,018.6
2	1,142.2	1,142.6
20	1,347.6	n.d.
13	1,582.8	1,582.4
7	2,100.2	2,099.5
5	2,199.6	2,198.6
14	2,254.6	2,253.6
10	2,583.8	2,583.4
22	2,854.3	2,853.7
19	4,596.2	n.d.

[a] The material was derived from 350 pmol of protein applied to the gel, and 10% of the column eluate was diverted to the MS and analyzed as described in Figure 1. The peptide masses were calculated using the sequence established by Sciaky et al. (1976). In peptides 2 and 10, the masses of the amides were chosen for the undiscriminated Asx and Glx, respectively.

[b] n.s., not scanned; n.d., not detected.

der acidic conditions. Previous quantitative experiments using radiolabeled proteins suggested close to quantitative recovery of peptides from the Immobilon CD membrane (Patterson et al., 1992). Furthermore, in a study using cyanogen bromide protein cleavage and characterization of recovered fragments by matrix-assisted laser desorption time-of-flight MS, we were able to detect all the predicted peptide fragments from different proteins, suggesting that complete recovery of all peptides from Immobilon CD is possible if optimal elution conditions are used (in prep.). Together, these data indicate that peptide maps generated by proteolytic cleavage of gel-separated and electroblotted proteins are comparable with peptide maps generated by fragmentation of the same protein in solution.

Chromatographic peaks in the TIC were clearly detectable if as little as 17.5 pmol of the intact protein, corresponding to 0.5 μg of CA, was applied to the gel, and peptide masses consistent with the masses predicted for tryptic peptides could be determined. This is illustrated in Figure 5. The mass spectra of peptide T5 displayed were derived from analysis of 17.5, 35, 175, and 350 pmol of CA, respectively, applied to the gel. It should be pointed out that the data permitted assessment of the purity of the eluting peptides even if submicrogram amounts of the protein were applied to the gel. By comparing the UV absorbances of protein digests in solution and on the membrane, we estimated that peptide amounts detected in the HPLC column eluate typically corresponded to a 30–60% yield relative to the molar amount of intact polypeptide applied to the gel. In the case of the 17.5-pmol sample, an estimated 0.8 pmol of peptide T5 was introduced to the MS, whereas the remainder (an estimated 8 pmol) was collected for Edman degradation or other analyses.

Analysis of protein isoforms separated by gel electrophoresis by LC/MS

One of the potential advantages of the LC/MS peptide mapping technique is the possibility of obtaining sufficient information for the comparison of proteins on the primary structure level without the necessity to determine partial or complete polypeptide sequences.

We applied the LC/MS separation/detection method to the characterization of isoforms of the enzyme ACC on the primary structure level. The major subunit of rat liver ACC exhibits an estimated M_r of 265,000, as determined from the published cDNA sequence (López-Casillas et al., 1988). Purified preparations of liver and mammary gland ACC subjected to SDS-PAGE also exhibit a slower-migrating, less abundant subunit/isozyme with an estimated M_r of 280,000 (Bianchi et al., 1990). The slower-migrating protein represents the principal form of ACC expressed in heart muscle (Thampy, 1989). We attempted to determine the structural basis for the observed difference in electrophoretic mobility using the LC/MS peptide mapping approach. It was anticipated that detailed analysis of the structures of peptides derived from the 265-kDa and the 280-kDa ACC isoforms would provide evidence to indicate if these proteins represented translation products of different genes, isoforms derived by alternative splicing, or differentially modified/processed products of the same gene.

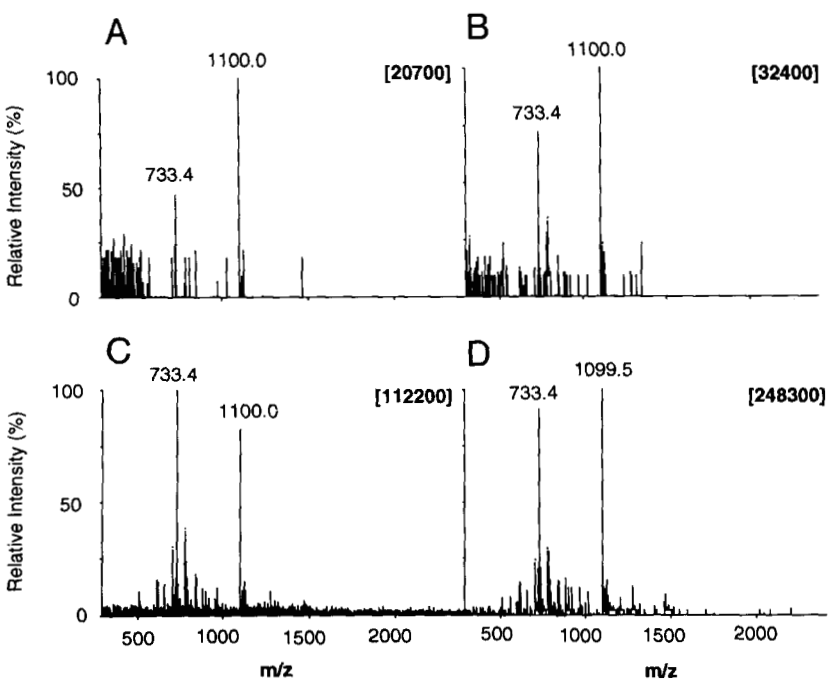

Fig. 5. Electrospray mass spectra of peptide 5 (Figs. 3, 4) at four different concentrations. The peptide was generated by tryptic cleavage of carbonic anhydrase electroblotted onto Immobilon CD. Ten percent of RP-HPLC column effluent was diverted into the MS. Samples (**A:** 17.5 pmol; **B:** 35 pmol; **C:** 175 pmol; **D:** 350 pmol) of the protein were subjected to SDS-PAGE. The signals of *m/z* 733.4 and 1,100.0 represent the $(M + 3H)^{3+}$ and the $(M + 2H)^{2+}$ ions, respectively, of the single peptide with a mass of 2,197.6 Da. The numbers in brackets are the combined total ion counts of the double and triple charged ions.

A mixture consisting of an estimated 300 pmol of the 265-kDa and 280-kDa rat liver ACC isoforms was reduced and carboxymethylated and subjected to electrophoretic separation by SDS-PAGE. Separated proteins were electroblotted onto Immobilon CD and cleaved on the support with Lys-C endoproteinase, and resulting peptides were separated by RP-HPLC. Figure 6 shows the TIC of peptide maps of a 10% aliquot directed into the MS (Fig. 6A, 265-kDa isoform; Fig. 6B, 280-kDa isoform). Comparison of the TIC traces and detailed mass analyses of all the recovered peptides indicated that the two polypeptides most likely represented translation products of different genes. With the exception of the major peptide eluting in fraction 37 of the 280-kDa polypeptide digest, which was matched exactly by the mass of a peptide eluting under comparable chromatographic conditions from the 265-kDa polypeptide, all the masses of the major peptides recovered from the two enzyme isoforms were different. Subsequent peptide sequence analysis of a number of collected peptides confirmed the data obtained by mass analysis alone. The sequence of peptide T37 isolated from the 280-kDa protein completely matched the published sequence of the 265-kDa protein (López-Casillas et al., 1988), whereas the sequences of all the other peptides analyzed showed differences between the primary structures of the two isoforms (R. Winz, D. Hess, R. Aebersold, & R.W. Brownsey, in prep.).

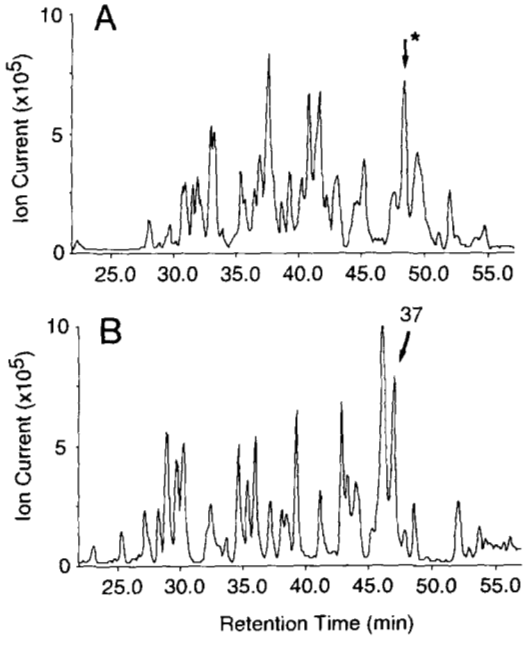

Fig. 6. Comparison of the peptides released by Lys-C digestion of the 265-kD (**A**) and 280-kD (**B**) isoforms of acetyl-CoA carboxylase. Samples of the two isoforms of acetyl-CoA carboxylase were separated, digested, and subjected to RP-HPLC and MS analysis as described above. The total ion current for all masses between 500 and 2,400 Da is displayed. The peptides marked with an arrow have the same mass in both isoforms.

Conclusions

In this paper we report the characterization by LC/MS of proteins separated by polyacrylamide gel electrophoresis. Proteins were extracted from polyacrylamide gels by electroblotting onto Immobilon CD membranes and enzymatically fragmented on the surface, and the resulting peptides were injected into a microbore chromatography system equipped with a 1-mm-i.d. column. Using a postcolumn flow-splitting device, a fraction of the eluting peptides (typically 10%) was diverted into the MS, and the remaining peptide samples were manually collected for further analyses such as liquid scintillation counting or peptide sequence analysis. Using this system, we were able to simultaneously obtain the molecular weights of the peptides from enzymatic digestion of the protein as well as purify the peptide fractions and recover 90% of the sample. This analysis has been achieved using as little as 17 pmol of protein applied to the gel. The technique is equally compatible with protein separation in one- and two-dimensional gel systems.

HPLC column flow rates in the range of 5 μL/min are also compatible with continuous-flow fast atom bombardment MS, and the use of CF-FAB for LC/MS peptide mapping experiments at sensitivities comparable to the ones reported here have been demonstrated (Caprioli et al., 1988; Henzel et al., 1990). Hemling and coworkers (1990) have pointed out several advantages of electrospray ionization compared to CF-FAB with respect to ease of operation, significantly extended mass range due to multiple charging, and generality, including detection of glycopeptides. An additional important advantage is that the requirement for external addition of matrix is eliminated in electrospray ionization.

The results presented here demonstrate that separation of polypeptides by SDS-PAGE and isolation by electroblotting can be achieved without significant chemical modifications of peptides or introduction of detectable levels of contaminants interfering with the electrospray MS analysis.

Although the experiments in this study were performed with the proteolytic enzymes trypsin or Lys-C, both of which generate fragments with basic C-terminal residues, the procedure is equally compatible with proteolytic enzymes of different specificity.

A method for the determination of peptide mass is essential for error-free interpretation of high-sensitivity peptide sequencing data. Mass analysis of RP-HPLC column effluents is currently the most sensitive and general method for evaluating peak purity, a parameter that is critical for the interpretation of peptide sequencing data. Important additional information can be deduced by comparing peptide mass determined in the MS with the value calculated from the sum of the amino acid residues determined by sequence analysis. Such a comparative analysis can unambiguously indicate whether the whole

peptide has been sequenced, confirms the deduced peptide sequence, and can be used to confirm ambiguous residues, thus drastically reducing the frequency of sequencing errors.

With the exponential growth of protein and DNA sequence databases, the emphasis of high-sensitivity protein analysis is shifting from determining partial or complete polypeptide sequences to correlating the primary structure of an isolated polypeptide with the respective entry in the sequence database. In combination with software that has been developed for the identification of sequence database entries based on predicted masses of proteolytic peptide fragments (Yates et al., 1991; Henzel et al., 1993; Pappin et al., 1993), the procedure described here now permits the rapid and sensitive identification at the primary structural level of proteins separated by one- or two-dimensional gel electrophoresis. Furthermore, through subtractive peptide mapping of differentially modified proteins, this technique is ideally suited for the high-sensitivity determination of the site and nature of post-translational modifications.

The approaches described here should be suitable for solving a wide variety of protein analytical problems and should find widespread application in the fields of protein biochemistry and cell biology.

Materials and methods

Materials

Reagents and solvents were of the highest available purity unless otherwise stated. Precast SDS-PAGE gradient gels (10–20% total acrylamide) were from Daiichi Pure Chemical Co. (Tokyo, Japan), Immobilon CD electroblotting membrane was from Millipore (Bedford, Massachusetts), Quick-Stain was from Zoion Research Inc. (Allston, Massachusetts), and fused silica capillaries were from Polymicro Tech. Inc. (Phoenix, Arizona). Proteins and enzymes were either purchased from Sigma (St. Louis, Missouri; carbonic anhydrase II, pI 5.9; bovine trypsin TPCK treated XIII) or from Wako (Dallas, Texas; *Achromobacter* protease I).

Instrumentation

Gel electrophoresis was performed on an Enprotech (Natick, Massachusetts) slab gel apparatus, and for electroblotting experiments we used the semi-dry Trans-Blot SD system from BioRad (Richmond, California). RP-HPLC peptide separations were done using an ultrafast microprotein analyzer (Michrom BioResources Inc., Pleasanton, California) equipped with a 300-Å Reliasil C18, 1 × 150-mm column provided by the same supplier. Mass spectra were generated with a model API-III triple quadrupole MS (Sciex, Thornhill, Ontario) equipped with an ion spray ion source (Bruins et al., 1987) for the provision of ion-evaporation mass spectra (Iribarne et al., 1983). The postcolumn flow splitter was a low dead volume splitter T from Valco (supplied by Sciex), and splitting ratios were tuned as illustrated in Figure 1 by adjusting the relative lengths and inner diameters of the fused silica capillaries to the MS ion source and to the fraction collector, respectively.

Electrophoresis, electroblotting, and protein detection

Proteins were separated using a precast 10–20% SDS-PAGE gel in a Laemmli buffer system. Following electrophoresis, separated proteins were electrotransferred to the Immobilon CD membrane using a buffer consisting of 48 mM Tris and 39 mM glycine at a pH of 9.2. The transfer buffer was supplemented with 1.3 mM SDS, and proteins were transferred for 40 min at 15 V. The Immobilon CD membrane was washed with water, and proteins were detected by staining using Quick-Stain according the directions of the manufacturer, except that the membrane was not washed between application of the staining and developing solutions and that a 2.5× higher concentration of the staining solution was used. Protein bands were cut out and either directly processed or stored frozen at −20 °C.

Electrophoresis, electroelution, and electroblotting of ACC

ACC was isolated according to a modification (R. Winz et al., in prep.) of the method by Brownsey et al. (1988) from Wistar rat liver. Proteins were reduced with dithiothreitol and carboxymethylated with iodoacetamide in the sample buffer prior to electrophoresis using standard protocols. Limited amounts of the 280-kDa and 265-kDa subunits of ACC were optimally separated by SDS-PAGE (4% T/0.66% C), and protein bands were visualized by staining without fixing in aqueous Coomassie brilliant blue R-250. The two separated isoforms were cut out individually, and the pooled bands from 10 separate lanes were electroeluted in an Isco 1750 electrophoretic sample concentrator (Isco, Lincoln, Nebraska) using a 40 mM Tris-acetate buffer system (pH 8.6) containing 2 mM EDTA and 0.01% SDS. The electroelution was conducted at constant power (3 W) for 1 h, followed by 1 W for 1 h. The isolated ACC subunits were then applied to a single lane for a second round of SDS-PAGE before electroblotting onto Immobilon CD. Protein detection on the membrane and proteolysis were done as described above and below, respectively.

Enzymatic cleavage of proteins on Immobilon CD

Tryptic cleavage

Protein bands were cut into small pieces and incubated in 10 μL of 0.1 M Tris, pH 8.2, containing 1 M NaCl,

10% MeCN, 2 mM $CaCl_2$, and 0.1 μg trypsin at 37 °C for 15 h, at which point 1 μL of saturated urea solution in water and an additional 0.1 μg of trypsin were added; incubation was then continued for an additional 6 h at 37 °C.

Lys-C (Achromobacter protease 1) cleavage

Protein bands were prepared as for tryptic cleavage and incubated at 37 °C in 10 μL of 0.1 M Tris buffer (pH 8.5) containing 1 M NaCl, 0.2 M urea, and 0.1 μg Lys-C. After 15 h, an additional 0.1 μg Lys-C was added and incubation was continued for another 6 h at 37 °C.

Enzymatic cleavage of proteins in solution

The proteins were cleaved under the same conditions as indicated above for the cleavage on Immobilon CD.

RP-HPLC separation of peptides

Peptide mixtures obtained by proteolytic cleavage on Immobilon CD were acidified with 1 μL of 10% trifluoroacetic acid. After removal of the primary supernatant, membrane pieces were extracted with 10 μL of 0.1% TFA in 10% MeCN, and the pooled supernatants were injected directly into the HPLC system. Peptides were separated by RP-HPLC using an ultrafast microprotein analyzer equipped with a 300-Å Reliasil C18 column with dimensions of 1 × 150 mm. Chromatography solvents were 0.05% TFA, 2% acetonitrile in H_2O (solvent A) and 0.045% TFA, 80% acetonitrile in H_2O (solvent B). The column was developed with a gradient from 5% to 50% solvent B in 30 min for the CA digests and from 5% to 50% solvent B in 60 min for digests of the two ACC isoforms. The flow rate was 50 μL/min, and UV absorbance was measured at 214 nm in a 200-nL flow cell with a path length of 2 mm.

HPLC-MS interface and postcolumn flow splitting

The instrument setup consisting of the API-III MS and a microbore HPLC system is illustrated in Figure 1. All the connections between the UV cell and the ion source consisted of fused silica capillaries with an i.d. of 50 μm. The connection between the flow splitter and the fraction collector was a fused silica capillary of 75 μm i.d. It was essential to use 50-μm-i.d. capillaries to maintain high resolution between the column and the ion source. The split ratio was set at the flow splitter by adjusting the lengths of the capillaries between flow splitter and ion source and between flow splitter and fraction collector, respectively. Fractions were collected manually according to the UV absorbance signal, taking into consideration the time delay between UV cell and the fraction collector (typically 5 s).

Ion spray MS analysis of peptides

All MS analyses were done by ion spray MS using an API III atmospheric pressure ionization tandem triple quadrupole MS. Peptides were analyzed in a matrix consisting of the HPLC effluent. The ion spray voltage was approximately 5,000 V and the nebulizer gas pressure was 40 psi. Flow rates into the ion spray inlet were 5–7.5 μL/min. All experiments were done in a single quadrupole operating mode using quadrupole 3 of the mass analyzer. The mass range from 300 to 2,400 Da was scanned with a step size of 0.5 Da and a dwell time of 1 ms per mass.

Peptide sequence and amino acid composition analysis

RP-HPLC separated peptides were collected manually for sequence analysis and applied to precycled polybrene treated glass fiber discs. Automated sequencing was done on a model 477A pulsed-liquid phase sequencer (Applied Biosystems, Foster City, California) equipped with a model 120A analyzer. Quantitative amino acid composition analysis was performed after acid hydrolysis of the peptide bonds in constant boiling HCl using the PTC precolumn derivatization method in a model 420A analyzer (Applied Biosystems) using standard chemical, chromatographic, and integration/quantitation protocols.

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