0026-895X/01/6003-405–415$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:405–415, 2001

Vol. 60, No. 3
765/928010
Printed in U.S.A.

MINIREVIEW

Mass Spectrometry of the Proteome

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Received December 19, 2001; accepted June 11, 2001

This paper is available online at http://molpharm.aspetjournals.org

Biological polymers undergo numerous significant and fascinating interactions, such as post-translational modifications, noncovalent associations, and conformational changes. A valuable parameter for the characterization of a biopolymer is molecular mass (MM). The field of mass spectrometry (MS) has evolved rapidly in the last decade through the introduction of two ionization techniques, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Modern methods of mass spectrometry, including ESI and MALDI, are ideally suited for accurate determination of the molecular mass of the biopolymers. Molecular weight measurements are now used routinely in the qualitative and quantitative analysis of macromolecules. In many cases, the small sample quantities (e.g., a few micrograms) limit the utility of nuclear magnetic resonance spectroscopy and X-ray crystallography for obtaining structural information. Thus, mass spectrometry, which may require only femtomolar or attomolar quantities offers an attractive alternative to the more traditional bioanalytical methods for rapid and sensitive measurements (Burlingame et al., 2000; Tomer, 2001). The ultimate goal of these experiments is to obtain sufficient information to map the complex molecular circuitry that operates within the cell (Aebersold and Goodlett, 2001; Godovac-Zimmermann and Brown, 2001). Herein, we present some of the aspects of the modern biological mass spectrometry for investigation of large molecules. For additional detailed technical descriptions, we have referred the reader to a number of recently published reports throughout the manuscript (see below).

MALDI and ESI Mass Spectrometry. The three main events during an MS analysis are ion production, ion transmission, and ion detection. In a typical MS experiment, the sample of interest is volatilized/ionized in the ionization source and guided via a series of electric and/or magnetic lenses to the detector. To control the motion of the ions during their transmission to the detector, it is necessary to control the influence of pressure and temperature on the ion mobility (kinetic energy). Therefore, a vacuum system with a pressure ranging from $\sim 10^{-5}$ to 10^{-8} Torr is used in all mass spectrometers. The vacuum environment minimizes the interfering collision of analyte ions with the background neutral gaseous molecules and facilitates their transmission to the detector.

A majority of commercial mass spectrometers use an electron multiplier detector, which provides an internally amplified electrical current subsequent to exposure to charged ions. The ion current output corresponding to each specific analyte is then processed by instrument electronics and translated to MM. In the resulting mass spectrum, the ordinate indicates the relative intensity or abundance, whereas the abscissa shows the observed ratio of mass to the number of charges on the ions. The latter is referred to as mass-to-charge ratio or m/z. The basic tenet is that regardless of the ionization source, the mass spectrometer measures the m/z.

MALDI (Fig. 1a) uses pulses of laser light to desorb the analyte from a solid phase surface (analyte cocrystallized with a light-absorbing matrix) and yield gaseous ions. Pulsed laser radiation, typically from a nitrogen laser (output wavelength, 337 nm) is used to initiate the desorption/ionization event and to simultaneously generate a packet of ions of different m/z values. The matrix is typically a small organic molecule (e.g., nicotinic acid, 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid) that has an absorption band that closely coincides with the energy of the laser radiation. The matrix is generally cocrystallized in large molar excess to the analyte; this facilitates the ionization of the analyte and minimizes the sample degradation from the laser radiation. For MALDI, the sample preparation procedure can be extremely crucial because the ion population depends upon the type of matrix and the presence of impurities. Depending on the specialist's experience and instrumentation, it is now possible to acquire MM information of a biopolymer using femtomolar to attomolar quantities of samples (Nelson and Krone, 1999; Nelson et al., 2000; Farmer and Caprioli, 1998). However, in some cases, additional sample quantities are required for detailed analyses such as peptide, polysaccharide, or oligonucleotide sequencing (Siuzdak, 1996; Dell and Morris, 2001).

This work was supported in part by National Institutes of Health Grant 1-R43-GM56603–01A2.

ABBREVIATIONS: MM, molecular mass; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; ESI, electrospray ionization; LC, liquid chromatography; CE, capillary electrophoresis; PAGE, polyacrylamide gel electrophoresis; 2-D, two-dimensional; EST, expressed sequence tag; DPD, double parallel digestion; 2-DE, two-dimensional electrophoresis; CID, collision-induced dissociation; CIEF, capillary isoelectric focusing; ICAT, isotope coded affinity tag; TOF, time-of-flight; MSIA, mass spectrometric immunoassay; β_2m, β-2-microglobulin; Eβ_2m, equine β-2-microglobulin; Hβ_2m, human β-2-microglobulin.

A complementary technique to MALDI is ESI (Fig. 1b), which produces single or multiply charged gaseous ions directly from a solution by generating a fine spray of highly charged droplets in the presence of a strong electric field. There are two widely proposed theories for ion formation in ESI. One theory suggests that ionized sample molecules are expelled from the droplets. Alternatively, it has been proposed that individual ionized sample molecules remain after continuous solvent evaporation and droplet fragmentation (Fernandez de la Mora et al., 2000).

As shown in Fig. 1b, a solution of analyte(s) and the solvent are introduced into a sampling metal capillary (~100 μm in internal diameter), which is charged with the application of an electrical voltage (4–5 kV). The voltage polarity of the metal capillary is positive or negative for positive or negative ion generation, respectively. At some point, the mutual repulsion between the ions at the surface becomes greater than the surface tension of the liquid. If the electrical field is sufficiently high, the spray commences and small charged droplets form. The ions generated by ESI carry multiple charges, provided the sample molecules have a MM of more than about 1000 Da. The characteristic feature of ESI that distinguishes it from other ionization techniques is that it generally imparts multiple charges to larger analyte molecules and the extent of multiple charging increases in near proportion with MM. The resulting highly charged molecular ions are thus within the *m/z* range in which most conventional mass spectrometers function quite well. It is the multiple-charging phenomenon that allows the assay of high-mass ions by mass analyzers with only a modest *m/z* range (Bakhtiar and Nelson, 2000; Bakhtiar and Tse, 2000; Thomas et al., 2000; Hofstadler and Griffey, 2001).

ESI does have limitations in that it is not very tolerant of the presence of salts, detergents, and inorganic buffers (MALDI has proven to be more amenable in such cases). Thus, to minimize signal suppression effects, ESI is often exploited as an interface between liquid chromatography (LC) or capillary electrophoresis (CE) and a mass spectrometer. Currently, LC-MS is an attractive tool in the analysis of complex mixtures in biochemical research and medical/diagnostic analysis. The up-front chromatographic separation aids in sample purification/enrichment from most common laboratory buffers and endogenous salts, and provides an additional useful parameter such as retention time.

Proteomics. The human body is estimated to contain ~35,000 protein-encoding genes (Lander et al., 2001). Furthermore, post-translational modification, mutation, degradation, and alternate RNA splicing routes dramatically increase the number of potential

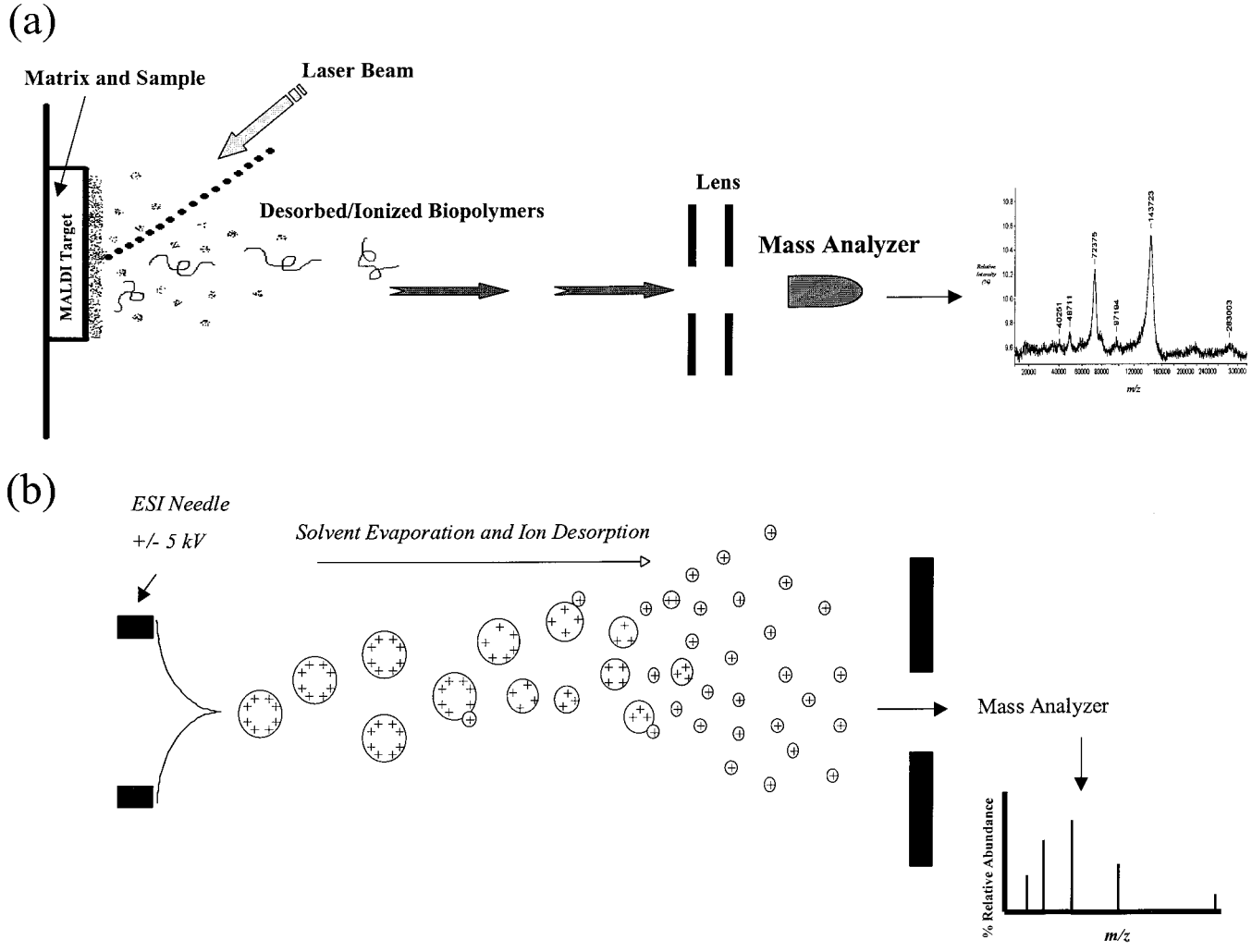

Fig. 1. a, in a MALDI experiment, the sample is mixed or dissolved with an excess amount (e.g., 1 part sample to 10,000 parts matrix) of a matrix component (having an absorption wavelength, which matches closely with the laser wavelength). Upon laser irradiation, a plume of neutral molecules and ions are desorbed. The ions are then guided to the mass analyzer and the detector by electrostatic lenses. In contrast to ESI, MALDI generally does not yield multiple-charged ions, does not require mass spectral de-convolution, and is more suitable for analysis of complex mixtures. b, a simplified schematic diagram of an ESI source operating in the positive ion mode. A spray of fine droplets that contains the charged analyte and solvent molecules is generated upon the application of a high electrical tension on a needle. The voltage polarity of the metal capillary is positive or negative for positive or negative ion generation, respectively.

cellular proteins. The science of proteomics involves the detection, identification, and characterization of proteins produced by the genome, thus providing a functional annotation for the entire proteome (Anderson et al., 2001; Gabor Miklos and Maleszka, 2001). The proteome is a highly dynamic system that can be influenced by environmental variations (stress), such as quantitative changes in protein expression caused by exposure to xenobiotics. The information obtained from proteome analysis can aid in identifying therapeutic targets or surrogate markers in understanding the initiation and progression of disease states. Thus, proteomics research can be a valuable tool in drug discovery and offers scientists an integration of genomics, mRNA analysis, protein expression, and protein-protein interactions.

The extremely high degree of complexity of multicellular organisms warrants the need for a collection of sensitive and rapid analytical techniques to yield qualitative and quantitative information with high efficiency and accuracy. One of the most commonly used techniques for protein separation has been based on gel electrophoresis. Simple protein mixtures (<100 components) are normally separated using one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE). On the other hand, for complex protein mixtures (e.g., cell or tissue extracts), the resolving power of a two-dimensional (2-D) SDS-PAGE may be required. In this approach, proteins are separated by an isoelectric point in the first dimension (preferably using immobilized pH gradient gels) and subsequently by their molecular masses in the second dimension. Visualization of the gel is achieved by staining, fluorescence tagging, or radioactive labeling, with some protocols having a detection limit of ~1 to 100 ng of protein (Gygi et al., 2000a; Rabilloud, 2000). However, visualization does not provide unambiguous protein identification; therefore, scientists typically use Western blotting or classical Edman sequencing for this purpose. Fortunately, the integration of the inherent benefits of MS (e.g., sensitivity, selectivity, and speed) with those conferred by protein and expressed sequence tag (EST) databases has led to a significant advancement in proteome characterization (Pandey and Mann, 2000; Yates, 2000; Mann and Pandey, 2001).

Figure 2 depicts a simplified strategy that is being used widely in high-throughput protein characterization. Typically, samples obtained from different cellular fractions are processed by 2-D SDS isoelectric-focusing gel electrophoresis. Each cell or tissue type may require a specific visualization approach such as Coomassie blue, silver staining, or fluorescence tagging for protein detection (Hancock et al., 1999; Williams, 1999; Lahm and Langen, 2000). Subsequently, gel images are electronically retrieved by high-resolution scanners and analyzed (spot-finding) using pattern recognition techniques against 2-D gel database queries (Miura, 2001). Sophisticated software packages can be employed to enhance contrast, subtract background, align images, remove artifacts, and perform gel comparison. Proteome maps are then compared against databases for identification of up- or down-regulation in a disease state. The resulting information can have the potential to identify biomarkers in clinical or toxicological studies (Alaiya et al., 2001; Bichsel et al., 2001).

Conceptually, an approach that is similar and complementary but has higher accuracy (i.e., better than 10 ppm), sensitivity, and speed can be implemented with the aid of MS-based techniques. The gel "spots" can be excised, washed, subjected to proteolytic digestion, and characterized by MALDI- or ESI-MS in an automated fashion (Neubauer and Mann, 1999; Pandey et al., 2000; Westergren-Thorsson et al., 2001). Strategies such as the yeast two-hybrid system,

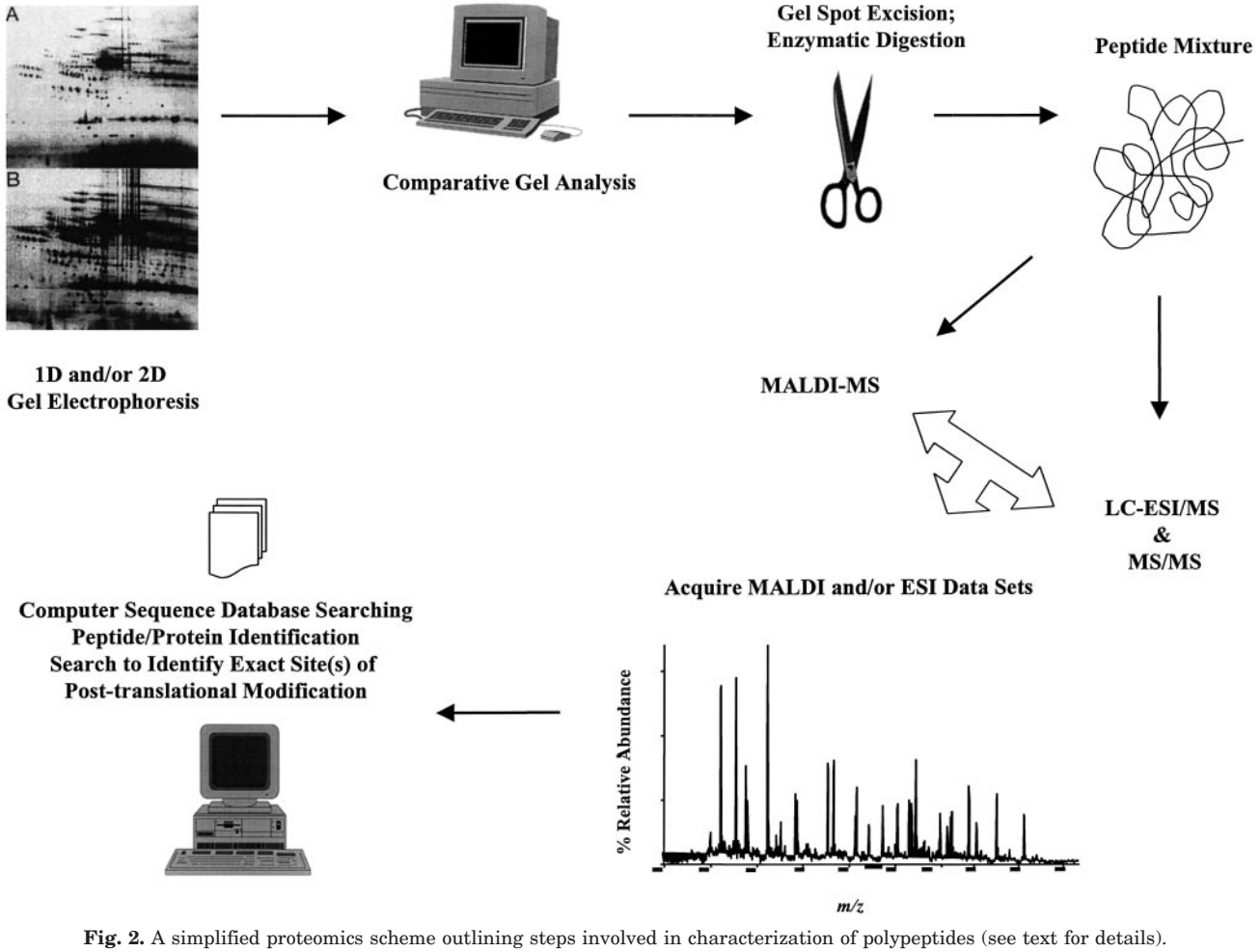

Fig. 2. A simplified proteomics scheme outlining steps involved in characterization of polypeptides (see text for details).

affinity chromatography techniques, or prefractionation steps may be necessary to target a specific class of proteins prior to additional sample manipulations (Link et al., 1999; Gruninger-Leitch et al., 2000; Husi et al., 2000; Zuo and Speicher, 2000). Commonly, on-line chromatography coupled to MS analysis can be adapted to further separate complex protein or peptide mixtures (Chong et al., 2001). A number of software packages are currently available to perform query in large databases (e.g., bioinformatics approaches) and enhance the speed of the MS protein identification process (Clauser et al., 1999). Several of these software algorithms for protein or gene identification are available via the World Wide Web (Fenyo, 2000). At the present, nonredundant protein databases with ~350,000 entries and human EST databases with ~1,200,000 entries can yield most sophisticated and accurate identification output compared with the 2-D gel analysis (Jensen et al., 1999). In addition, 2-D gel sample components below MM of ~10,000 Da or above 100,000 Da that are not easily characterized can be scrutinized readily by MS. The following examples will clarify the above discussions.

Recently, a high-throughput protein identification [double parallel digestion (DPD)] method was reported by Sanchez and coworkers (Bienvenut et al., 1999). In the DPD approach, partially digested proteins were obtained using an immobilized trypsin membrane and subsequently transblotted. The resulting peptides were trapped on a polyvinylidene difluoride membrane and scrutinized by MS. The DPD approach was successfully applied to a mini-2D gel electrophoresis of *Escherichia coli* extract. A potential drawback of 2-DE-MS approach is its limited utility in the analysis of low-abundance (copy-number) or poor solubility proteins. This is particularly an issue in higher eukaryotic cells, which tend to be complex and contain a host of proteins with diverse isoelectric points and MM. Therefore, to achieve a meaningful visualization, the recommendations are: make improvements in the protein recovery steps (e.g., prefractionation, affinity LC), perform subcellular fractionation and purification of organelles, and/or start with larger amounts of proteins (Gygi et al., 2000a; Jung et al., 2000). Alternatively, elegant on-line multidimensional or tandem liquid chromatography-mass spectrometry strategies have been proposed as a substitute for the more widely used 2-D gel analysis (Davis et al., 2001; Washburn et al., 2001). These methods involve mixed bed microcapillary columns containing strong cation exchange, size exclusion, and/or reversed phase resins. The tandem LC approach offers the potential to avoid the need for time-consuming 2-DE and dramatically increases the number of peptides that can be identified in a complex mixture. In addition, it offers a wide dynamic range and alleviates the protein solubility problems encountered during 2-DE experiments, because the proteins undergo enzymatic digestion *en masse* (Haynes and Yates, 2000).

Post-translational modification of proteins plays a pivotal role in functional activity and signal transduction in all living organisms. The mass changes caused by post-translational modifications (i.e., acetylation, farnesylation, glycosylation, phosphorylation, methylation, sulfation) can be easily detected by ESI or MALDI (Knotts et al., 2001; Merrick et al., 2001; Nemeth et al., 2001). Identification of the protein fragment of increased mass after enzymatic digestion allows possible determination of the site of modification. For example, in the case of phosphorylation, radiolabeling with ^{32}P is not required and low levels of phosphopeptides (i.e., 200–300 femtomoles) can readily be identified with high efficiency and speed. Additional information is discerned by performing tandem mass spectrometry or collision-induced dissociation (CID) experiments. In a typical CID experiment, a beam of ions with a specific *m/z* (denoted as the precursor or parent ion) is selected and collided with a neutral and nonreactive gas phase target such as argon. These collisions result in subsequent fragmentation and product ions that are a direct consequence of dissociation of the precursor ion. Generally, the resulting fragmentation pattern is unique for all ions having a particular structure. CID experiments are particularly useful in partial or complete peptide sequencing (Baldwin et al., 2001; Oda et al., 2001). In this approach, low-energy CID experiment is used to generate spectra dominated by product ions resulting from cleavage at the amide bonds. Because the low-energy CID yields very little amino acid side-chain fragmentation, the interpretation of the CID spectra becomes relatively simple. In high-throughput proteome analysis, the observed fragment ions are often matched against the predicted fragment ions of all peptides in a database (Aebersold and Goodlett, 2001; Zhou et al., 2001). A series of scores are then assigned to each "hit" to rank the quality of the match. Clearly, this approach is useful for genetically well-characterized organisms in which substantial protein or cDNA sequence exists.

Figure 3 depicts the MS and tandem MS (MS/MS) of a synthetic cyclic phosphopeptide in positive and negative ion mode, respectively. The hexapeptide, cyclo[-Gly-Tyr(PO_3H_2)-Val-Pro-Met-Leu-], corresponds to the autophosphorylation segment around Tyr^{751} of the platelet-derived growth factor receptor β subunit. This peptide serves as an inhibitor of the receptor tyrosine kinase/*scr* homology region 2 domains that mediate mitogenic signal transduction pathways (Barchi et al., 1996). After liquid chromatography or direct infusion of the sample, the molecular mass of the phosphopeptide is determined by ESI-MS in positive ion mode (Fig. 3a). To ascertain the presence of post-translational modification, the MS is switched to negative ion and the precursor or parent ion scanning mode. In this mode of operation, the phosphopeptide is fragmented using tandem mass spectrometry and yields a signature ion with high specificity at *m/z* 79 (i.e., loss of PO_3^-). Consequently, the resultant simplified mass spectrum exhibits only precursor ions that lost *m/z* 79 (Fig. 3b). This technique is particularly useful in detecting peptides containing phosphotyrosine residues, which are difficult to discern from the background chemical noise in a typical single stage ESI-MS experiment (Wilm et al., 1996; Vener at al., 2001). A similar strategy could be applied for the detection of glycopeptides containing *N*-hexosamines yielding characteristic ions at *m/z* 204 and *m/z* 168, corresponding to loss of the oxonium ion of *N*-hexosamine and the oxonium ion with a double water loss, respectively (Wilm et al., 1996). Recently, an alternative specific approach involving the detection of the immonium ion of phosphotyrosine at *m/z* 216.043 in positive precursor ion scanning mode combined with high-resolution quadrupole time-of-flight MS has been described (Steen et al., 2001).

Another related area in proteome analysis is protein-expression mapping, which is defined as the quantitative measurement of the protein dynamics in the specimen (e.g., cell, tissue, or body fluid) of interest. In this approach, the proteome analysis is typically performed in a subtractive fashion whereby the alterations in individual proteins for two or more states are compared. These so-called "cell-states" could refer to a cell before and after treatment/stress with xenobiotics or cells obtained from normal and pathological states. Protein-expression mapping is of interest in the early drug development (preclinical safety) stages and toxicological studies when exposure, effects, and susceptibility in laboratory animals are performed.

Although the de novo identification (qualitative analysis) of cellular proteins using MS technology is becoming widely recognized, the quantitative cellular stress response measurements provide an added benefit of the extent to which an organism is altered. In this regard, Smith and coworkers (Pasa-Tolic et al., 1999) demonstrated the utility of ultra-high resolution MS measurements in conjunction with the resolving power of capillary isoelectric focusing (CIEF) for characterization of cadmium stress response in *Escherichia coli* K-12 strain MG1655 cells. The cells were cultured in normal as well as rare-isotope (e.g., ^{13}C, ^{15}N, ^{2}H) depleted media to provide internal standards for all detected proteins after cadmium-mediated stress. Abundance of several intact and isotopically distinctive proteins was monitored qualitatively for up to several hours using this method. Presumably, this type of stable-isotope approach eliminates the need for the 2-DE based analysis of crude mixtures by adapting on-line CIEF-MS. However, the above procedure is not easily amenable to the characterization of proteins in tissues. Furthermore, stable-isotope enriched media tend to be costly. Lastly, the protein identifica-

tion must be performed before quantification to decipher the incorporation of stable isotopes within the specific sequence of protein.

Recently, an elegant quantitative microcapillary-LC-ESI-MS strategy for the analysis of protein mixtures in yeast *Saccharomyces cerevisiae* was reported by Aebersold and coworkers (Gygi et al., 1999, 2000b; Gygi and Aebersold, 2000; Griffin et al., 2001; Ideker et al., 2001). An isotope coded affinity tag (ICAT) that consisted of an affinity tag (biotin), a linker containing stable isotopes, and a reactive moiety with propensity to react with free sulfhydryl groups (e.g., cysteines) was used (Fig. 4). Two sets of cell states (or tissue extracts) were independently treated with the isotopically light and heavy (8 Da higher in MM by incorporation of ^{2}H) ICAT reagents. The cells were combined and subjected to proteolytic cleavage. The ICAT-labeled peptides were isolated using the biotin tag and analyzed by microcapillary-LC-ESI-MS. Peptide sequence information was obtained by tandem mass spectrometry experiments and identified by computer searches against protein data banks. Quantification of proteins was performed by ratios of respective light and heavy ICAT-labeled peptides, which were generated using enzymatic digestion. The stable isotope labeling procedure was a clever approach to assist in the identification of two peptides with identical sequences and molecular mass values from two different cell-states. Because all the physical characteristics of two identical protein samples from two cell-states remain the same, the resulting peptide fragments obtained from enzymatic cleavages yielded identical mass spectra (Fig. 4). Thus, incorporation of specific stable isotopes in one cell-state resulted in mass shifts, which in turn served as an internal standard for all other cell-states within the same experiment. Because the light and heavy ICAT tagged peptides were chemically identical, one can safely assume that they would yield analogous MS detection (ionization) response and behave as mutual internal standards for quantification purposes. Other similar procedures such as biotinylation of cysteine containing peptides (Spahr et al., 2000), N-terminal labeling using light and 1-([H_4/D_4]nicotinoyloxy)succinimide esters

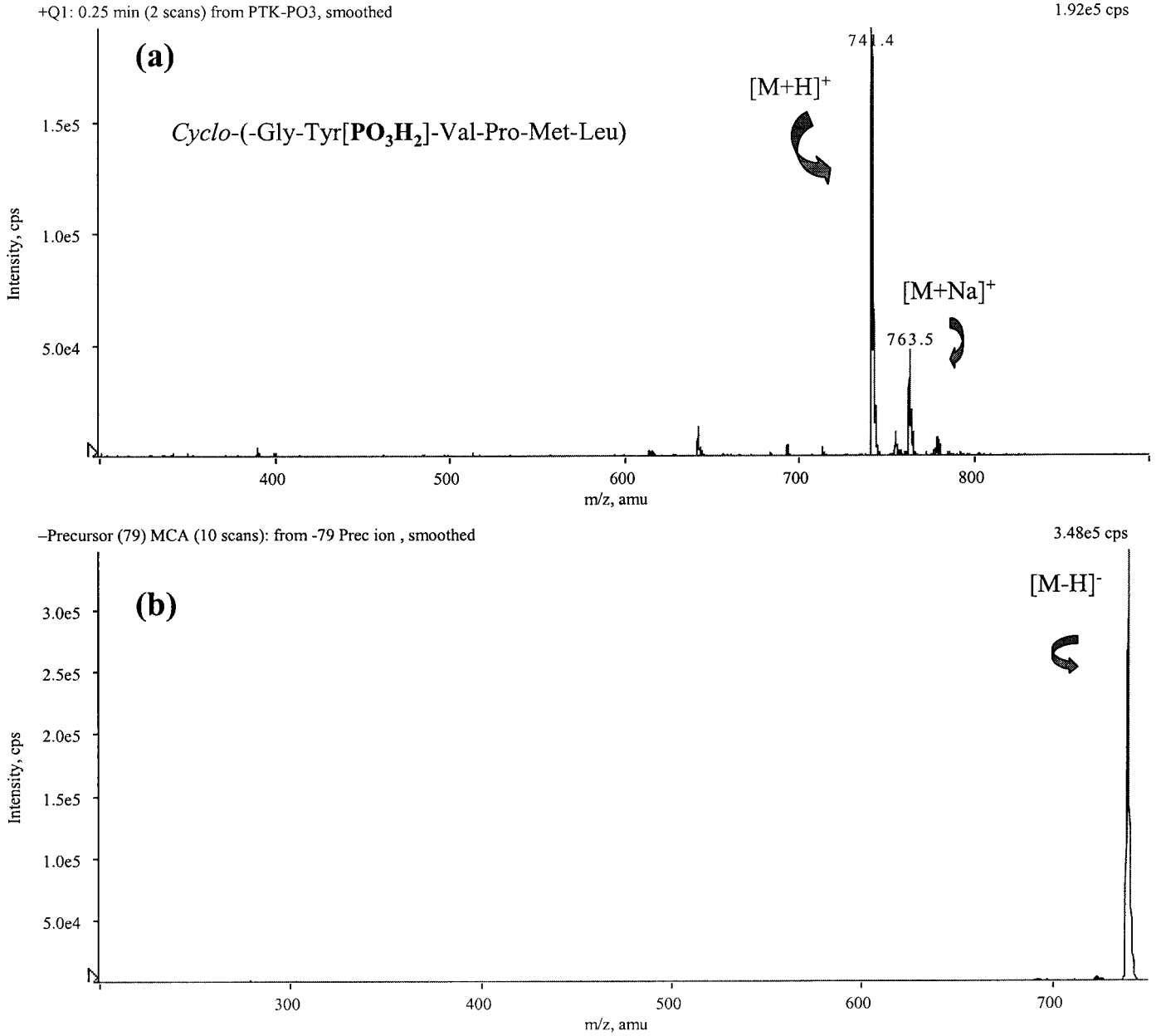

Fig. 3. a, positive ESI mass spectrum of the hexapeptide, cyclo[-Gly-Tyr(PO_3H_2)-Val-Pro-Met-Leu-] using a triple quadrupole mass spectrometer yielding signals at *m/z* 741.4 and *m/z* 763.5 corresponding to $[M+H]^+$ and $[M+Na]^+$ ions, respectively; b, parent ion scan for *m/z* 79 (loss of PO_3^-) in negative ion mode for the same peptide. The phosphopeptide is clearly evident in its singly charged form at *m/z* 739.5 corresponding to $[M-H]^-$ ions.

(Munchbach et al., 2000), and derivatization of primary amine moieties with acetate and trideuteroacetate (Ji et al., 2000) have recently been reported. Currently, a drawback of the ICAT strategy is its limited scope to the cysteine containing proteins. This limitation should be alleviated with the introduction of ICAT reagents specific for other abundant amino acids.

In general, the above protocols for qualitative and quantitative analysis of the key cellular proteins could aid in several areas of drug discovery and development such as toxicological and pharmacogenomics studies. For example, in the science of pharmacogenomics, genetic polymorphisms in transporters, drug-metabolizing enzymes (e.g., cytochromes P450, uridine 5′-triphosphate glucuronosyltransferases), receptors, and therapeutic target proteins have been postulated to be one of the underlying reasons in variable responses to drug treatments in patients (Broder and Venter, 2000; March, 2000; McLeod and Evans, 2001). Target validation that could predict a well-tolerated medicine with adequate efficacy in humans is a widely perceived challenge. Currently, these investigations are rather tedious and empirical. In most cases, the human genetic variations resulting in great heterogeneity in response to medications are realized in large studies at the postmarketing stages (i.e., population sizes exceeding 100,000). Some of these idiosyncratic responses are toxic and thus it is essential to identify them before phase III clinical studies (Gould Rothberg et al., 2000; Knowles et al., 2000). According to a *meta*-analysis published in the *Journal of the American Medical Association* (Lazarou et al., 1998), adverse or idiosyncratic drug reactions in hospitalized patients is one of the major causes of mortality. Consequently, it is of interest to elucidate the identity and pharmacogenomic traits (i.e., polymorphically expressed enzymes) of key cellular proteins and to design optimum medication for individual patients. MS technology offers a viable platform that can be utilized to assay differential protein expression after drug treatment.

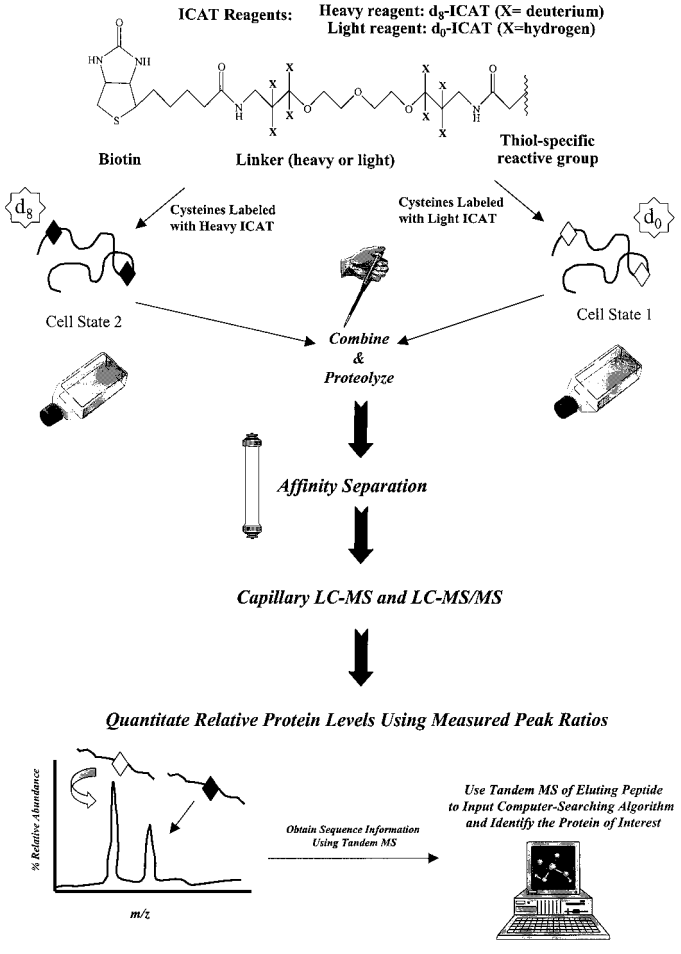

Fig. 4. Principle of quantitative proteomics by incorporation of a stable isotope-labeled tag. Cysteine amino acids of proteins in two different cell states (i.e., normal versus abnormal due to a stress cause by a drug or other environmental factors) are covalently modified by the tag. One tag contains ^{2}H and the other incorporates ^{1}H. The protein extracts from the two cell states are mixed, digested by trypsin, and separated by affinity chromatography. The resulting peptide mixtures are then subjected to LC-MS and tandem mass spectrometry. The ratios of labeled and unlabeled peptides (different by 8 Da) is the manifestation of the abundance of the gene in the two cell states. Subsequent, LC-MS/MS peptide sequencing experiments of these peptides can identify the gene product, which is being quantified. A computer-search algorithm that accesses large protein data banks is typically used during the identification process.

Characterization of Clinical Biomarkers. The need and prospects for new and improved biomarkers in therapeutic area as clinical assessment tools are evident. It can be argued that once a biomarker is identified, it is more efficient to monitor its structural permutations and/or expression level by direct assay rather than the global 2-DE approach. Thus, there is still much impetus to develop technologies for detailing protein structure and accurately monitoring protein expression levels. Over the past 8 years, we have devoted much effort to developing devices and methods for the rapid MALDI-TOF analysis of proteins residing in complex biological media. Our approach, termed mass spectrometric immunoassay (MSIA) (Nelson et al., 1995) is shown in Fig. 5. In general, analytes are retrieved selectively from solution by repetitive flow through a receptor-derivatized porous frit constructed at the entrance of a wide-bore P-200 pipettor tip (MSIA-Tip). Once washed of the nonspecific compounds, the retained species are eluted onto a mass spectrometer target in preparation for mass spectrometry. MALDI-TOF then follows, with analytes detected at precise *m/z* values. Analyses are qualitative by nature but can be made quantitative by incorporating mass-shifted variants of the analyte into the procedure for use as internal standards (Nelson et al., 1994, 1995; Wang et al., 1996; Rudiger et al., 1999). The approach is ideally suited to high-throughput applications using robotics and automated MALDI-TOF instrumentation. Recently, we have explored the benefits of using the MSIA approach in structural characterization and quantification of β-2-microglobulin (β_2m), a low-molecular-mass protein, identified as the light chain of the class I major histocompatibility complex. Full details of the approach are given elsewhere (Tubbs et al., 2001). Briefly, anti-β_2m polyclonal antibody MSIA-Tips were used in selective extraction of β_2m from various human biological fluids. Figure 6 shows MALDI-TOF and MSIA spectra obtained from a human urine sample. The MALDI-TOF spectrum shows a number of signals in the peptide region and an absence of signal for β_2m. The MSIA spectrum is dominated by signals from the β_2m, with few additional signals from nonspecified components.

Protein quantification using MALDI-TOF requires use of internal standards to compensate for varying laser intensities and spot-to-spot differences in sample composition that give rise to fluctuations in analyte ion signal (Nelson et al., 1994). Although proteins with characteristics unlike those of the analyte may be used as internal standards (as has been shown during protein quantification directly from mixtures (Nelson et al., 1994; Muddiman et al., 1995) or during MALDI-TOF quantification of affinity-retrieved species by addition of an internal reference standard to peptides eluted from beaded affinity reagent (Kuwata et al., 1998), internal reference standards that behave similarly to the analyte during laser desorption/ionization are generally preferred. This prerequisite is met during MSIA by choosing internal references that share sequence homology with the target protein: enzymatic/chemically modified versions of the targeted protein (Nelson et al., 1995; Krone et al., 1996; Wang et al., 1996), truncated/extended recombinant forms of the target proteins, the (same) target protein recombinantly expressed in isotopically-enriched media (e.g., ^{15}N or ^{18}O), or the same protein from a different biological species. Given that the receptor is able to capture both the target protein and the internal reference, MSIA can be designed

around a single receptor system (Nelson et al., 1995; Wang et al., 1996). Alternatively, a two-receptor system can be considered where one receptor is used to retrieve the target protein and a separate receptor is used to retrieve the internal reference (Nelson et al., 1995; Krone at al., 1996; Rudiger et al., 1999).

The internal reference chosen for this study was equine β_2m (Eβ_2m), which shares ~ 75% homology with its human counterpart (Hβ_2m) and is ~300 Da less in mass than Hβ_2m (thus, both species share similar characteristics and are easily resolved in the mass spectra). Although, no data could be found on the relative dissociation constants between the polyclonal anti-β_2m IgG and Hβ_2m or Eβ_2m, preliminary studies showed that the antibody exhibited cross-reactivity sufficient to retain both species. Figure 7a shows spectra representing MSIA analyses of Hβ_2m standards in a concentration range of 0.01–1.0 mg/l. Each spectrum, normalized to the Eβ_2m signal, is one of 10 65-laser shots spectra taken for each calibration point. Plotting the average of the 10 normalized Hβ_2m integrals for each standard versus the Hβ_2m concentration results in the working curve shown in Fig. 7b. Linear regression fitting of the data yields $I_{H\beta 2m}/I_{E\beta 2m}$ = 4.09 mg/l Hβ_2m + 0.021 (R^2 = 0.983), with a working limit of detection at a S/N > 3 of 0.0025 mg/l (210 pM) and a limit of quantification of 0.01 mg/l (850 pM). The standard error of all points of the working curve is ~ 5%.

Urine samples were collected from four persons over a 2-day period: female, 31 years (F31); male, 30 years (M30); male, 36 years (M36); and, male 44 years (M44). All of the subjects were in a state of good health when the samples were collected. Results from MSIA of the urine samples are summarized in Fig. 7 (inset). The bars depict the β_2m concentration determined for each sample, and the inset spectra above each bar shows the respective Hβ_2m signals normalized to Eβ_2m. The data for the samples show remarkable consistency, with an average β_2m concentration of 0.100 ± 0.021 mg/l (high = 0.127 mg/l; low = 0.058 mg/l). An additional analysis was performed on a urine sample obtained from an 86-year old female (F86) who had recently suffered a renal infection, revealing a significantly higher β_2m concentration (3.23 ± 0.02 mg/l).

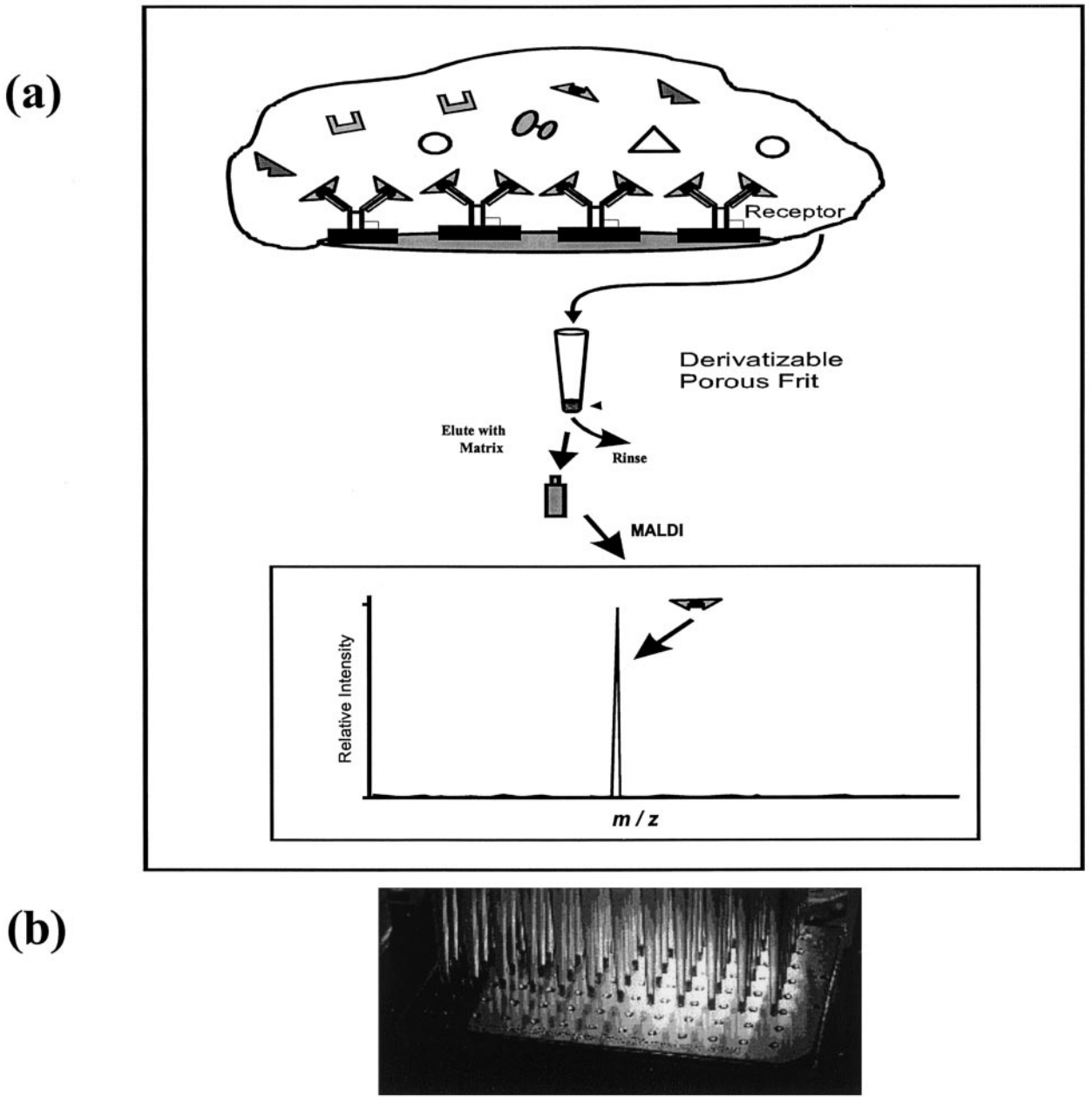

Fig. 5. a, illustration of the MSIA procedure. b, MSIA applied with robotics for high-throughput analyses.

The mass-selective detection of MSIA makes possible the discovery and quantification of variants of β_2m that may be present in urine. During quantitative screening of the urine samples, a second, higher molecular mass species (Δm = +161 Da) was coextracted with

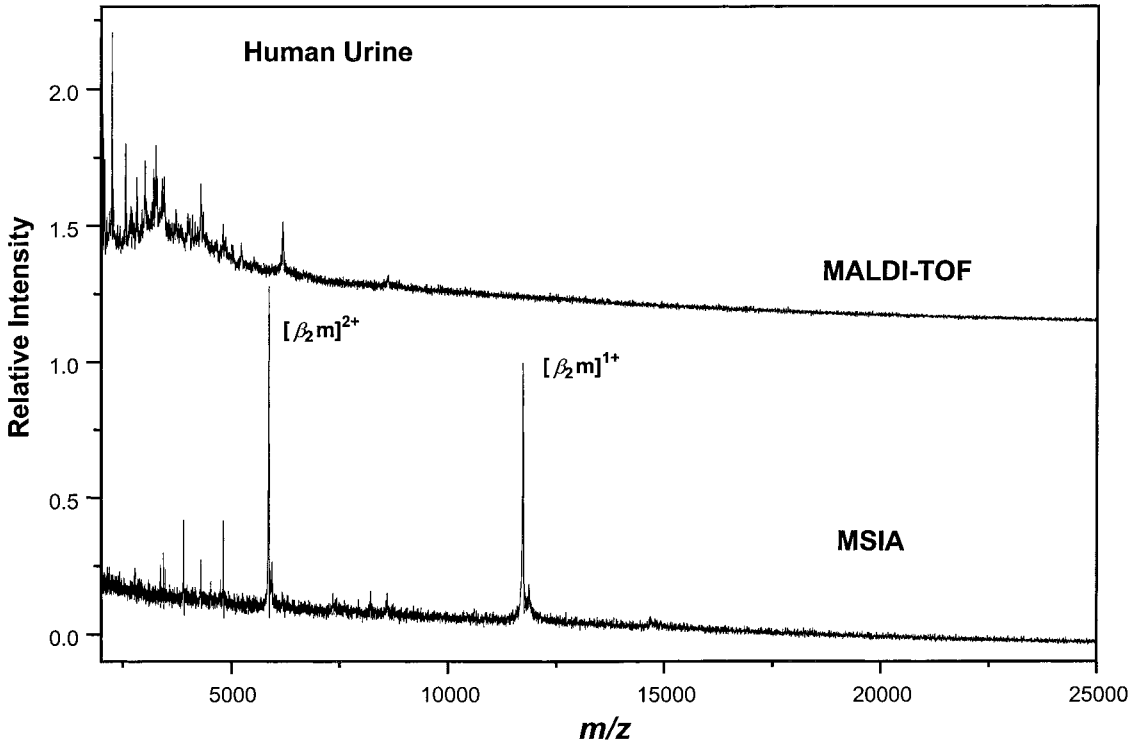

Fig. 6. β_2-microglobulin MSIA screening of urine. Samples were prepared by dilution of urine in preparation buffer and repetitive flow incubation through the MSIA-Tip. Tips were washed using buffer and water before elution of retained compounds directly onto a mass spectrometer target using α-cyano-4-hydroxycinnamic acid (saturated in 1:2 acetonitrile/water and 0.2% trifluoroacetic acid). β_2m is observed as the predominant signal in the MSIA spectrum, whereas direct MALDI-TOF of urine yields little useful information.

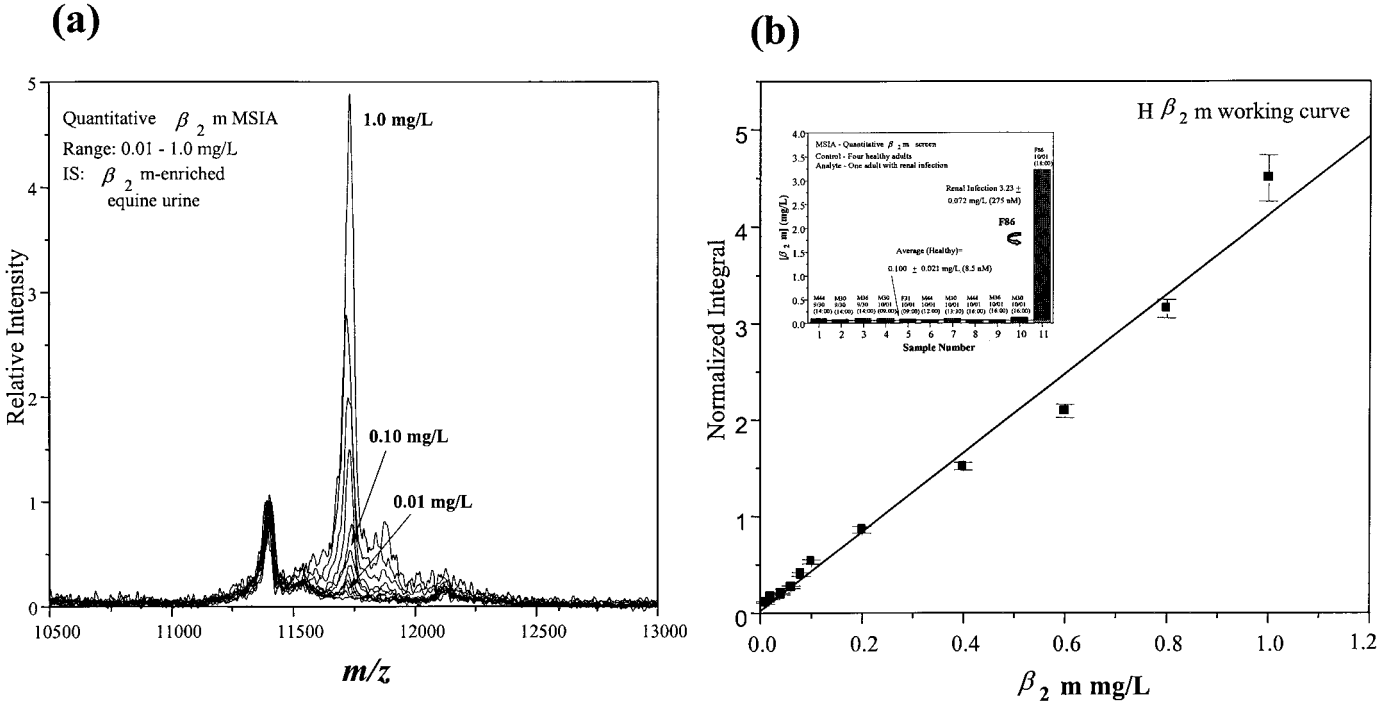

Fig. 7. Quantitative β_2m-MSIA. a, representative spectra of data used to generate working curve. Human β_2m concentrations of 0.01 - 1.0 mg/l were investigated. Equine β_2m (MM = 11,396.6 Da) was used as an internal standard. b, working curve generated using the data represented in a. The 2-decade range was spanned with good linearity (R^2 = 0.983). Error bars reflect the S.D. of 10 repetitive 65-laser shot spectra taken from each sample. Inset, β_2m- screening. Urine samples from five subjects were screened over a period of 2 days. The average value determined for healthy persons was 0.100 ± 0.021 mg/l. The level determined for an 86-year old female (F86) with a recent urinary tract infection indicated a significant increase in β_2m concentration (3.23 ± 0.072 mg/l).

the β_2m. The species is presumably a glycosylated (one hexose) form of β_2m and is observed most prominently in F86. Figure 8 shows an overlay of two MSIA spectra taken from the urine of F86 (diluted × 20) and M36 (no dilution; given for comparison). The level of glycosylated β_2m is much greater in F86 than in M36. The specific cause of the elevated level of the glyco-β_2m, which is roughly the same concentration as wild-type β_2m in the urine samples from the healthy subjects, is at present uncertain. Note that because the two β_2m species are differentiated using mass spectrometry, the concentration of the wild type β_2m determined during MSIA accurately reflects the concentration of only the wild type β_2m and not the combination of both of the species. Thus, MSIA holds a particular advantage over other techniques that are unable to differentiate between similar forms of a target analyte. Elevated β_2m levels are used as a general indicator of immune system activity, whereas β_2m-glycosylation has been associated with more specific ailments [e.g., advanced glycosylated end-products associated with dialysis related amyloidosis (Niwa, 1997)]. MSIA is able to deconvolute these independent contributing factors and yield results that more accurately relate a specific biomarker with a specific disease state.

Conclusion and Future Outlook. During the past decade, dramatic progress in the field of mass spectrometry has resulted in a large increase in the number of commercially available MS instruments. Based on the overwhelming number of published manuscripts, it is clear that MS is becoming a pivotal bioanalytical tool in many biotechnology and biochemistry laboratories. MALDI- and ESI-MS allow the characterization of a myriad of small and large molecules with high sensitivity, speed, accuracy, and efficiency. MS-based techniques are becoming a permanent component of studies involving functional genomics, proteomics, early drug discovery, clinical diagnostics, and combinatorial chemistry.

Although the applications shown here have focused on protein quantification, there are numerous other applications in which the MSIA-Tips can be used in the qualitative (structural) characterization of proteins. In general, tips can be derivatized with affinity ligands and used for retrieval of proteins from mixtures. Screening of expression systems for production of the correct construct is a good example. Because most cloning vectors incorporate some general utility affinity tag into a recombinant protein, MSIA-Tips derivatized with antibodies toward these tags (e.g., anti-His-tag or anti-*myc* antibodies) can be used to selectively retrieve recombinant proteins from expression systems, after which MALDI-TOF is used to verify production of the correct protein via accurate mass determination. Alternatively, tagged-peptides containing nascent sequence of an expressed protein can be used to identify the expressed protein (Nelson et al., 1999). Such an application stands to find use in identifying components of DNA libraries that would otherwise be identified at the DNA level (through hybridization approaches or sequencing). Chelate surfaces can also be used to selectively retrieve metal-binding polypeptides for rigorous mass spectrometric characterization, as has been shown in the recent independent works of two laboratories (Posewitz and Tempst, 1999; Qian et al., 1999). Finally, in further-reaching applications, receptors may be immobilized for use in selective retrieval of receptor-interacting proteins from solution under native conditions. MSIA-Tips can be used in these applications to feed directly into MALDI-TOF or ESI instrumentation, or alternatively into one-dimensional gels, if further separation of retained compounds is necessary (Neubauer et al., 1998). These applications promise to complement protein-interaction reporter techniques such as yeast two-hybrid systems or instrumental techniques such as surface plasmon resonance-based biosensors with an emphasis, however, on the protein structural-analysis (e.g., identification) capabilities of mass spectrometry.

The future prospects of mass spectrometry are exciting. Advancements in miniaturization and chip technology using photolithography/etching fabrication (Nelson et al., 2000; Figeys and Pinto, 2001; Kodadek, 2001), identification of single-nucleotide polymorphisms (Griffin et al., 1999; Stoerker et al., 2000; Buetow et al., 2001), clinical diagnostic applications (Worrall et al., 2000; Chace, 2001), rapid enzyme kinetics analysis, (Bothner at al., 2000; Gross et al., 2000; Norris et al., 2001), and further adaptation of automation/robotics sample processing steps (Lopez et al., 2000; Kyranos et al., 2001) will continue to have a significant impact on modern medicine.

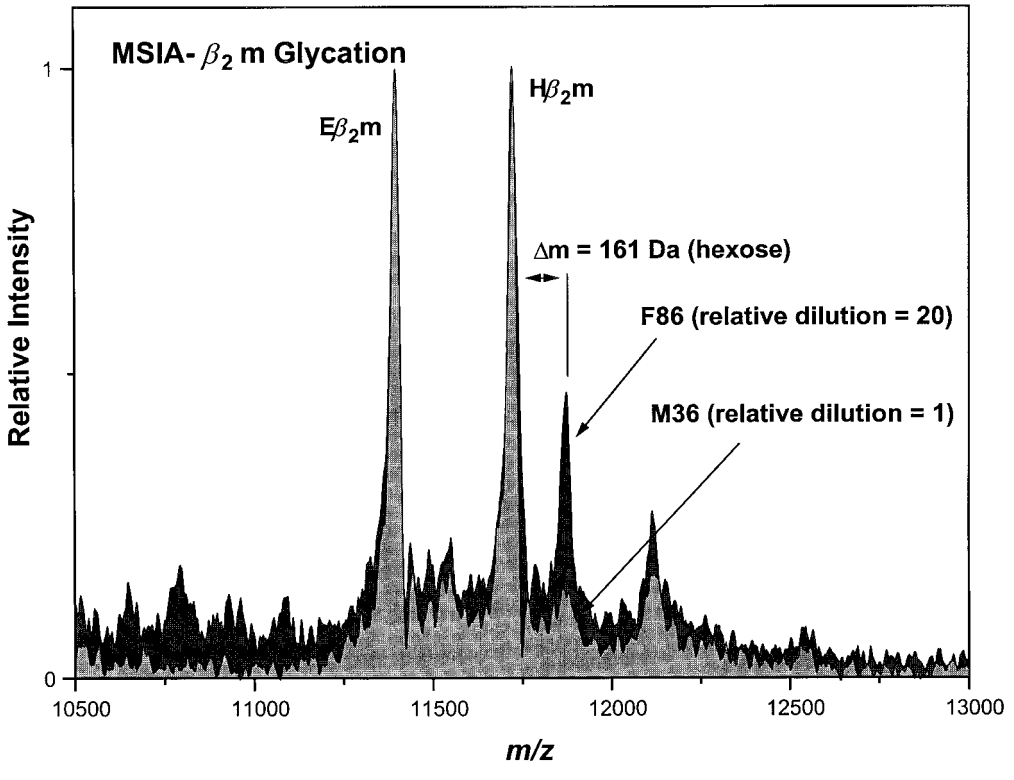

Fig. 8. MSIA showing elevated level of β_2m-glycan in 86-year-old female (F86, dark gray). During MSIA, a second signal is observed at Δm = +161 Da, indicating the presence of glycosylated β_2m. MSIA is able to adequately resolve the two β_2m forms resulting in a more accurate quantification of the nascent β_2m, and possible quantification of the glycan. Such differentiation is important considering that the two β_2m forms originate from (or are markers for) different ailments. MSIA of a healthy individual, showing little glycation, is given for comparison (light gray).

Acknowledgments

R.B. thanks the Finnigan Corporation (San Jose, CA, USA) for providing Fig. 1b.

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