Part 7

Genes of Metabolism: Generating, Sustaining, and Modifying the Machinery of Energy Metabolism. Regulation of Gene Expression for Metabolic Enzymes

7.1 Proteomics

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Abstract: Proteomics is the study of the proteome defined as the set of all proteins of a cell, an organ, or the whole organism. Currently, the proteomic approach usually combines two-dimensional (2D) gel electrophoresis and mass spectrometry (MS) as a common and powerful approach. The specific feature of proteomics is the simultaneous detection of many proteins involved in different biochemical pathways. With regard to the brain, this approach has proven to be particularly beneficial for the analysis of energy metabolism and cellular respiration. To gather proteomic information, techniques were developed to handle the large amount of data yielding a comprehensive pattern of protein alignment within brain biochemical pathways. It is expected that in future proteomics will become a routine tool in all aspects of brain cell biology and beyond those of bioenergetics, including cell development, differentiation, and cell death. Proteomics then will complement other approaches to analyze cerebral function and biochemistry under normal and disease conditions.

1 A Profile of Proteomics

1.1 Proteomics: Complement to Genomic Approaches

The term "genome" describes the whole set of genes, whereas the term "proteome" encompasses the protein complement of the genome (Wasinger et al., 1995). It subsumes the whole set of proteins of a cell, an organ, or the whole organism. The study of the proteome, called proteomics, is even more comprehensive and subsumes not only all proteins in a given compartment, but also the respective protein isoforms and modifications, as well as the information on their structure and interaction (Tyers and Mann, 2003). The genome remains relatively constant during the lifetime of an individual, but the proteome undergoes constant changes as a reaction to environmental influences on protein synthesis. Specifically, proteins are modified posttranslationally by phosphorylation, glycosylation, the addition of carbohydrate chains (methylation, acetylation), proteolytic cleavage, sulfation, and by many other biochemical reactions resulting in various isoforms of a specific protein (Mann et al., 2001; Mann and Jensen, 2003). In addition, the rate of synthesis and degradation of a specific protein can vary, resulting in different concentrations of the same protein under different conditions. Thus, the proteomic analysis represents only a "snapshot" of the pattern of protein expression at a given point of time in a specific environment.

In the context of functional genomic approaches, the proteomic analysis complements the set of diverse approaches, which include gene expression profiling using microarray technology; systematic phenotypic analysis of cells and organisms (phenomics) based on structural, ultrastructural, and functional aspects; and systematic genetics using mutational analysis and RNA interference (Tyers and Mann, 2003). Additionally, one gene and its transcript mRNA may encode for more than one protein. For example, in humans, one gene encodes for more than ten protein isoforms on an average (Gunning et al., 1998; Kim et al., 2004). Additionally, posttranslational modifications contribute to the diversity of protein isoforms (Mann and Jensen, 2003). Although there is no doubt that the levels of DNA, RNA, and protein are highly related and interconnected, several studies have found only limited correlation between transcriptomic and proteomic data (Anderson and Seilhamer, 1997; Link et al., 1997; Gygi et al., 1999b; Lee et al., 2003). Besides technical variability of the molecular screening methods applied, biological divergences contribute to the explanation of these findings: molecular half-life, synthesis and decay rates, mutations, reaction kinetics, environmental stress, cell cycle, disease, and multigenic and epigenetic influences are all factors influencing the concentrations of cellular RNA and of protein species (Humphery-Smith et al., 1997). Therefore, proteomics can be seen as an additional and complementary tool besides other exploratory data analysis approaches such as differential display, high-density nucleic acid arrays, expressed sequence tags, hybridization techniques, chromosomal linkage studies, and nucleic acid sequencing.

In principle, two main areas in the field of proteomics have been developed, each of them having its pros and cons. These fields are "profiling" and "functional" proteomics (Choudhary and Grant, 2004). The aim of proteomic profiling is to describe and index the whole set of proteins of a biological sample, which could be an organism, an organ, or a cell, or parts thereof like individual tissues or organelles. Profiling also

includes differential protein expression levels under specific experimental conditions or the comparison of different types or origins of sample material. Thus, proteomic profiling describes the inventory of proteins at a particular point of time. In contrast to the more static approach of proteomic profiling, the term functional proteomics encompasses direct functional aspects, like enzyme activity, protein interactions, and posttranslational modifications (PTMs). Although these two experimental approaches cannot be seen completely separate, profiling has been regarded to be of minor biological relevance due to its descriptive nature. However, such an opinion does not seem justified, since the cataloging of existing proteins is a basis to generate new hypotheses, which trigger further biological investigations. On the other hand, functional proteomics is based on protein profiling, since one needs to know which proteins to search for when it is intended to focus on a subset of proteins that are functionally coupled. In our understanding, both types of proteomics are valuable tools complementing other biological methodologies that help to describe the complexity of nature, and, specifically, brain function.

1.2 Proteomics as a Screening Tool in the Neurosciences

In general, proteomics can be used for a wide variety of applications, including (1) validation of genome sequences, (2) identification of novel proteins, (3) characterization of regulating (stimulating and inhibiting) proteins, (4) detection of posttranslational modifications, (5) monitoring expression patterns of large sets of proteins, (6) high-resolution protein purification, (7), detection of immunogenic proteins, e.g., in vaccine studies, (8) analysis of mechanisms of action of therapeutic agents and their toxicological relevance, and (9) identification of novel drug targets (O'Connor et al., 2000).

With regard to neurological disorders, we have to take into account the complexity of these diseases. The etiology and pathogenesis of many neurological diseases are not well defined at a molecular level. When investigating potential changes in protein expression as a basis of brain disease, several limitations have to be taken into account. The amount of deviating proteins may be less, thus obviating the detection of relevant proteins. Many details of protein interactions as well as of signaling and metabolic pathways are still not understood. In addition, the diseases themselves may not show well-defined protein targets. For example, the players in Alzheimer's disease appear to be known, but the debate of what is cause and what is epiphenomenon is still not solved. For schizophrenia, several candidate markers are discussed, but their interrelation is unknown. Proteomic technology may open new doors in the search for biomarkers and their pathophysiological interactions.

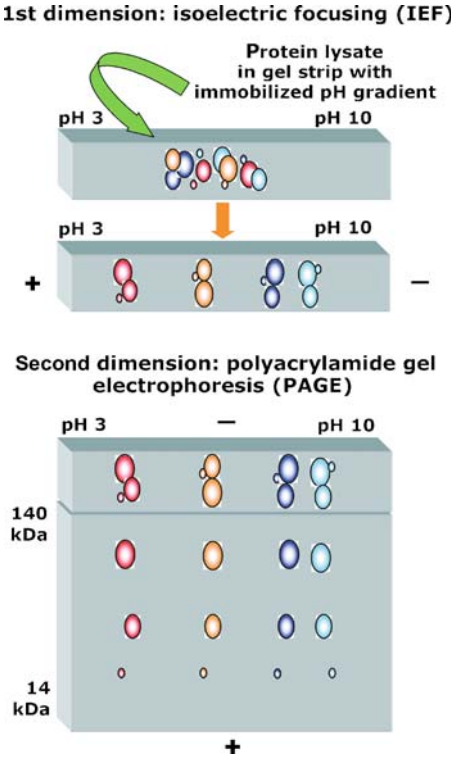
Proteomic applications in the neurosciences have been proposed for a variety of fields, including learning and memory, brain injury, ischemia, addiction, neurodegenerative diseases, polyglutamine repeat disorders, depression, anxiety, bipolar disorders, epilepsy, and brain tumors (Rohlff, 2000; Morrison et al., 2002; Kim et al., 2004). In the near future, the techniques of proteomics will be increasingly used for the development of novel pathophysiological concepts, novel diagnostic and prognostic markers, and novel therapeutic strategies.

2 Techniques

2.1 Two-Dimensional Gel Electrophoresis

The majority of proteomic results was obtained using a system of two-dimensional (2D) gel electrophoresis to separate proteins according to their isoelectric points in the first dimension and according to their molecular weight in the second dimension (*Figure 7.1-1*). This approach allows a high-resolution separation and quantitation of at least several hundred or even thousands of proteins or polypeptides. The method is limited by the fact that only the most abundant proteins can be detected and the proteins have to be present within the given pH and molecular mass range (Görg et al., 2000; Rabilloud, 2002). The rapid development of new applications in the field of mass spectrometry (MS) has favored direct mass spectrometric approaches (Tyers and Mann, 2003), but a complete substitution of 2D gel electrophoresis by

Figure 7.1-1
Principles of two-dimensional (2D) gel electrophoresis. In the first dimension, solubilized proteins are separated by isoelectric focusing (IEF) in a gel containing an immobilized pH gradient. After high voltage is applied, the proteins migrate to their respective isoelectric points. The gel strip of the first dimension is then attached to a polyacrylamide gel. In the second dimension, the polyacrylamide gel sieve separates the proteins according to their respective molecular weights



MS is still not possible (Dutt and Lee, 2000; Herbert et al., 2001; Ong and Pandey, 2001; Rabilloud, 2002; Patterson and Aebersold, 2003).

A typical 2D gel electrophoresis experiment consists of several subsequent steps (Freeman and Hemby, 2004):

1. sample preparation,
2. first dimension: isoelectric focusing,
3. second dimension: polyacrylamide gel electrophoresis,
4. in-gel staining of proteins,
5. digitizing of gel images and image analysis, and
6. protein identification.

In the following sections, we will describe some of the most common principles and protocols of these steps.

2.1.1 Sample Preparation

Principles of sample preparation. Sample preparation is considered the most delicate and essential step to obtain reliable 2D gel electrophoresis results (for discussion of procedures and components, as well as

additional references, see Link, 1999; Berkelman and Stenstedt, 2001; Westermeier, 2001). The aim of sample preparation is to solubilize, disaggregate, denature, and reduce the proteins in a given biological sample in order to resolve any kind of binding to other proteins or compounds of cell membranes and organelles. Ideally, the proteins should then be freely dissolved in the sample buffer.

To obtain single molecule components out of a complex mixture of proteins, it is necessary to remove all possible interacting components such as protein complexes formed with membranes, nucleic acids, or other proteins. Moreover, although unspecific aggregates must be disrupted, the precipitation of proteins under these nonphysiological conditions must be avoided. The effectiveness of solubilization depends mainly on the cell disruption method, protein concentration, choice of detergent, and composition of the sample buffer. Detergents in the sample buffer are necessary for solubilization, disaggregation, and denaturation, whereas reducing agents prevent postextraction modifications of the proteins.

Different methods of pretreatment of the samples may be employed, according to the specific application desired (Patton, 1999). For the detection of intracellular proteins, cells must be completely disrupted. Proteases should be inhibited effectively, as they are released during this process. Reagents interfering with the subsequent electrophoresis steps such as salts, small ionic molecules, ionic detergents, nucleic acids, polysaccharides, lipids, and phenolic compounds must be removed by using, for example, ultrafiltration, microdialysis, gel filtration, or precipitation/resuspension. For the analysis of subcellular compartments and organelles, differential fractioning methods like ultracentrifugation have to be applied. A special challenge is the preparation of membrane proteins. Although membrane proteins are of great interest in cell signaling research, they remain the stepchild of proteomic analysis, because sample preparation of membrane proteins is poorly compatible with current isoelectric focusing (IEF) techniques.

Cell disruption methods. Mechanical and chemical approaches can be applied to achieve cell disruption. The method of disruption should depend on the cell type of interest. For cultured cells and microorganisms, a gentle lysis method like osmotic lysis, freeze–thaw lysis, detergent lysis, or enzymatic lysis may be chosen. For cells in solid tissues and plant cells containing cell walls, more vigorous methods like sonication, French pressure cells, grinding, mechanical homogenization, and glass bead homogenization may be helpful. Differential centrifugation steps may follow in order to isolate the cell organelles of interest.

Practical application. The typical sample buffer is composed of urea (and desirably, thiourea), zwitterionic detergents like 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), reducing agents like dithiothreitol (DTT), and carrier ampholyte buffer. For the preparation of proteins, extracts from brain cell cultures including neurons, astrocytes, and neural stem cells are obtained. These cells are dissolved in a detergent lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) Triton X-100, 0.5% (v/v) IPG buffer pH 3–10 (Amersham Biosciences, Uppsala, Sweden), 100 mM DTT, and 1.5 mg/ml complete protease inhibitor (Roche, Mannheim, Germany) for 1 h at room temperature in an orbital shaker (Maurer et al., 2003b, 2004). Twice the volume of lysis buffer is added to the cell pellet. For whole brains from mice (0.5 g) or rats (2 g), 2 ml of lysis buffer is recommended. The lysate should be centrifuged at 21,000*g* for 30 min. Protein content of the supernatant can be measured by the Bradford assay (Bradford, 1976), as this protocol is compatible with the major components of the lysis buffer (Ramagli, 1999). The supernatant should be stored at −80°C until further processing, and repeated freeze–thaw cycles should be avoided.

2.1.2 First Dimension: Isoelectric Focusing

Proteins carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The pH value at which the net charge equals zero is called the isoelectric point (pI). IEF allows separating proteins according to their isoelectric points. The presence of a pH gradient in the electrophoresis gel is essential to achieve the separation of proteins in the sample, which causes proteins to move in the electric field through the pH gradient until they reach their isoelectric point. If a protein diffuses away from the isoelectric point, it will gain charge and immediately be driven back. This is the essence of the focusing

effect. A milestone in IEF technology was the introduction of immobilized pH gradient gels (Görg et al., 1988, 1998), which allowed to establish comparable and reproducible pH gradients.

The resolution depends on the slope of the pH gradient and the electric field strength. Typically, IEF is performed at high voltages (more than 1,000 V). Once the proteins have reached their final position in the pH gradient gel, there is only little ionic movement, resulting in low currents (<1 mA). Normally, a constant number of volt-hours is applied for reasons of comparison within a series of experiments, defined by the integral of the volts applied over the time.

For initial screening experiments, one may choose a pH range of 3–10 to get an overview of the total protein distribution. To study the protein pattern in greater detail, or if only a certain pH range is of interest, "zoom" gels with a narrow pH range of 1 pH unit or even less are available.

For analytical brain and cell culture experiments, a typical IEF protocol includes running 250–500 μg of the protein extract in 6 M urea, 2 M thiourea, 1 M DTT, 2% (w/v) CHAPS, and 0.5% (v/v) IPG buffer on 18 cm immobilized nonlinear pH 3–10 gradient IPG strips (Immobiline DryStrip pH 3–10 NL, Amersham Biosciences, Uppsala, Sweden), using the IPGphor apparatus (Amersham Biosciences, Uppsala, Sweden). Other pH ranges may be used, for example, "zoom gels" may resolve a single pH step. Nowadays, pH gradient gels are distributed on plastic supports to ensure easier handling and protection of the gels, which are 3 mm thick when rehydrated. After 12 h of reswelling time at 30 V to remove disturbing salts, voltages of 200, 500, and 1,000 V are applied for 1 h each. Then voltage is increased to 8,000 V within 30 min and kept constant at 8,000 V for 12 h, resulting in a total of 100,300 Vh (Maurer et al., 2003b, 2004). After an IEF protocol, the gels may be stored at -80°C until further use.

2.1.3 Second Dimension: Polyacrylamide Gel Electrophoresis

In the second dimension, a polyacrylamide gel electrophoresis (PAGE) is performed under denaturating conditions in the presence of sodium dodecylsulfate (SDS) to separate proteins according to their molecular weight. The cross-linked acrylamide polymers act as molecular sieve; thus smaller proteins migrate faster than larger ones. The intrinsic charge of the proteins does not influence electrophoresis, as the anionic detergent SDS will provide negative charges for all proteins, thus the separation is based primarily on the molecular size. The most common buffer system used for the second dimension was described by Laemmli (1970), which resolves proteins at high pH and allows the detection of relatively heavy proteins up to a molecular weight of about 250 kDa. The Tris-tricine system of Schägger and von Jagow (1987) may also be used for resolution of smaller proteins and peptides below 10 kDa.

Before running the second-dimension gels, the pH gradient gels must be equilibrated for the new running conditions. Typically, the equilibration solution contains 2% (w/v) SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, a trace of bromophenol blue, and 100 mg/10 ml DTT to preserve the reduced state of the proteins, preventing postextraction modifications of the proteins. The reducing agents improve protein solubility during IEF, which results in shorter run times and increased resolution as well as decreased horizontal streaking on the subsequent second-dimension gel. A recent study replaced DTT by tributyl phosphine for even better solubilization (Herbert et al., 1998). The first-dimension gels are incubated for 20 min. In a second equilibration step, the gels are incubated for another 20 min in a buffer where DTT is replaced by 250 mg/10 ml of the alkylating agent iodoacetamide (IAA) to prevent reoxidation, which is necessary for maintaining solubility in the second-dimension gel electrophoresis.

For the second dimension, large gels should be used to resolve several hundreds or even thousands of protein spots. Good results can be achieved using $20 \times 20 \times 0.3$ cm^3 gel volumes. Klose and coworkers have developed the large-gel 2D electrophoresis (2DE) (Gauss et al., 1999; Klose et al., 2002), where the gel running length is 40 cm or more, but the gels are more difficult to handle than smaller ones. The final polyacrylamide concentration may vary between 5% and 20%, or gradient gels may be used, depending on the molecular size range that is to be resolved. Excellent step-by-step protocols have been described by Berkelman and Stenstedt (2001) and can be downloaded free of charge from the support section of http://www.amershambiosciences.com.

2.1.4 Staining Proteins in 2D Gels

After second-dimension SDS-PAGE, the separated protein spots are visualized in the gels. Most staining protocols originally developed for SDS gels can also be applied to second-dimension gels (Rabilloud, 2000). The optimal staining procedure should have (1) a high sensitivity, (2) a wide linear range for quantification, (3) an intrinsic compatibility with MS, and (4) low toxicity of the dye. Unfortunately, none of the existing staining procedures fulfill all of these requirements. On the other hand, a myriad of different staining methods and protocols has been developed based on the following:

1. Organic dyes like Coomassie Brilliant Blue R-250, Xylene Cyanine Brilliant G (better known as Coomassie Brilliant Blue G-250), Amido Black 10S, Procion Blue RS, Ponceau S, Alcian Blue, and Fast Green FCF (Wirth and Romano, 1995). The stains are mostly compatible with MS, although the methods described below are more sensitive.
2. Metal ions like silver (Blum et al., 1987; Rabilloud, 1999), zinc, and copper. Only some staining protocols allow full compatibility with MS (Shevchenko et al., 1996), although modified staining protocols show an excellent sensitivity.
3. Fluorescent dyes like SYPRO Ruby, SYPRO Orange, Cy3, and Cy5 (Patton, 2000a, b), which are compatible with MS and exhibit a sensitivity comparable with silver staining. Different excitation and emission wavelengths make these dyes well suited for differential proteome analysis of two samples in the same gel (DIGE; Ünlü et al., 1997; Ünlü, 1999).
4. Radiolabeling using the isotopes ^{14}C, ^{35}S, ^{32}P, ^{3}H, and ^{125}I (Wirth and Romano, 1995). Radioactive isotopes are mostly used for metabolic labeling during protein synthesis and still permit the most sensitive staining procedure. These methods require the incorporation of the isotope into living cells and therefore cannot be applied to body fluids, biopsy material, and other clinically available material from patients. Although radiolabeling can be applied to brain cell cultures and living animals, its use with regard to the human brain is limited and has not been described.

Table 7.1-1 compares the most common stains with regard to their detection limit and compatibility with MS.

Table 7.1-1
Comparison of the most common protein staining methods in 2D gels

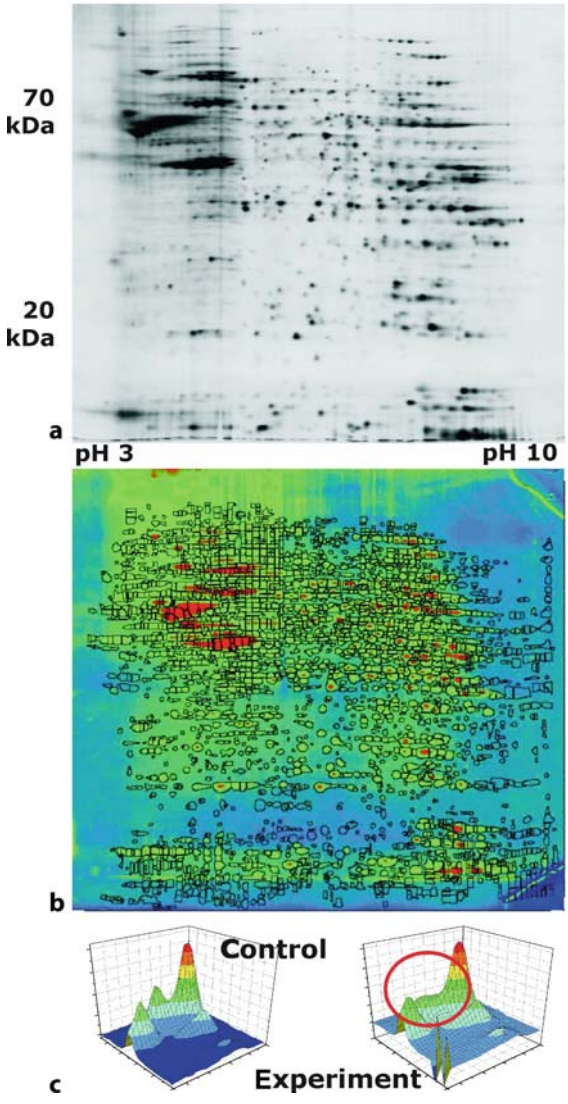
Dye	Detection limit (ng)	Compatible to MALDI-MS
Coomassie Brilliant Blue R-250	>100	Yes
Blum's zinc/imidazole	>100	Yes
SYPRO Orange	>30	Yes
Coomassie Brilliant Blue G-250	>30	Yes
SYPRO Ruby	>10	Yes
Silver	>10	No
Silver + glutaraldehyde	>1	Some protocols

2.1.5 Image Analysis and 2D Gel Electrophoresis Software

In a typical 2D experiment, the gel image analysis steps after staining include (1) digitizing the gels, (2) spot detection and quantitation of spot parameters such as the optical density or the volumes for each protein spot, (3) comparison of different gels, and (4) data analysis (*Figure 7.1-2*).

To this end, the stained 2DE gels are scanned and the digitized gel images subjected to image analysis. For digitizing, densitometers, CCD cameras, or laser imaging devices can be used. For proteomic experiments, specialized software is necessary to store and structure the huge amount of data. Sophisticated algorithms provide the basis for spot detection and quantitation of protein spots and intergel comparison (Garrels, 1989; Appel et al., 1997; Seillier-Moiseiwitsch et al., 2002; Maurer, 2004b).

Figure 7.1-2
Principle of two-dimensional (2D) gel analysis. (a) After protein separation, the proteins are visualized by a highly sensitive staining procedure such as silver staining. (b) Gel images are digitized and protein spots are detected by a special 2D analysis software. (c) For quantitative analysis, protein spot volumes, defined as the integral optical density of the spot area, are compared by statistical methods



In principle, spot detection relies on the accurate discrimination of the spot boundaries. A common problem is a low signal-to-noise ratio, mainly because of high gel background. Then, the gel background is subtracted and all gels in an experiment are normalized, either by external standards or by the total protein load. In the next step, gel matching is applied to geometrically align spots from different gels by mathematical algorithms (Garrels, 1989; Appel et al., 1997; Maurer et al., 2004), based on a procedure called polynomial image warping. First, an initial reference gel is chosen and several landmarks are selected on this reference image. Then corresponding spots in the experimental gel images are aligned to the reference.

Some of the software specified in *Table 7.1-2* requires time-consuming manual matching, whereas others provide fully automated spot detection and matching. A helpful tool integrated in some of the 2D software is the three-dimensional representation of gel spots, which allows easier detection of the spot limits and simplified spot matching (Maurer, 2004b).

Table 7.1-2
Commercially available 2D analysis software (as of September 2004)

Name	Distributor	Internet URL
DeCyder Differential Analysis Software	Amersham Biosciences, Uppsala, Sweden	www.amershambiosciences.com
Delta2D	DECODON, Greifswald, Germany	www.decodon.com
Expressionist Pro	GeneData, Martinsried, Germany	www.genedata.com
Gellab II+	Scanalytics, Fairfax, VA, USA	www.scanalytics.com
ImageMaster 2D Platinum 5.0[a]	Amersham Biosciences, Uppsala, Sweden	www.amershambiosciences.com
ImagepIQ	Proteome Systems, Sydney, Australia	www.proteomesystems.com
Investigator HT Analyzer	Genomic Solutions, Ann Arbor, MI, USA	www.genomicsolutions.com
PDQuest 2D	BioRad, Hercules, CA, USA	www.proteomeworks.bio-rad.com
Phoretix 2D	Nonlinear Dynamics, Newcastle-upon-Tyne, UK	www.nonlinear.com
ProteinMine	Scimagix, San Mateo, CA, USA	www.scimagix.com
ProteomWeaver	Definiens Imaging, München, Germany	www.proteomweaver.com
Z3	Compugen, Tel-Aviv, Israel	www.2dgels.com

[a]The program Melanie has been incorporated into the ImageMaster 2D Platinum series only recently. Melanie has been one of the leading two-dimensional (2D) gel electrophoresis software for many years and was developed by the Swiss Institute of Bioinformatics (SIB, http://www.isb-sib.ch)

Of note, online tools are available for comparison of 2D images over the Internet like Flicker (http://www.lecb.ncifcrf.gov/flicker) (Lemkin, 1999) and GelScape (http://www.gelscape.ualberta.ca) (Young et al., 2004), although these programs lack automated spot detection.

A typical 2D analysis software package should include algorithms for:

1. automated spot detection with numbering, annotation, and spot filtering,
2. background subtraction,
3. calculation of geometric spot characteristics such as area, optical density, or spot volume,
4. 2D calibration for isoelectric points and molecular weight,
5. averaging multiple sample gels,
6. choosing a reference gel and modifying the reference gel by adding spots,
7. matching spots in different sample gels,
8. normalization of spots for intergel comparison,
9. statistical analysis of the results,
10. directing spot picking devices,
11. including additional data such as mass spectrograms, and
12. creating a Web-based federated database to publish the data on the Internet.

Currently available programs are given in *Table 7.1-2*. Special care should be taken to guarantee the reproducibility of the gel analysis (Mahon and Dupree, 2001; Choe and Lee, 2003) as multiple software parameters can influence the quality of the analysis.

Several studies have compared commercially available 2D gel electrophoresis programs (Raman et al., 2002; Rosengren et al., 2003) by a standard set of gel images for parameters like spot detection, gel matching, and spot quantitation. These gel images can be downloaded from the URL http://www.umbc.edu/proteome and may be used for benchmarking the 2D software in the user's own lab.

2.2 Protein Identification

For protein identification, the protein spots stained and mapped on the 2D gels are excised. Manual spot cutting was used in the beginning. Later, the advantages of automated spot cutting became obvious: There is only a minimal risk of contamination of the samples, the access to the individual protein spots is highly precise, data may be automatically tracked, and these systems are well suited for high throughput applications. Therefore, manual spot picking should be avoided.

Several methods for protein identification have been developed including MS as the gold standard, amino acid sequencing, immunoaffinity identification, and protein arrays. In this chapter, we describe the most widely applied techniques for the identification of 2D gel electrophoresis spots.

2.2.1 Mass Spectrometry

Recent years have shown tremendous advances in mass spectrometric methodology and instrumentation. For the identification of proteins by MS, sensitivity, resolution, and mass accuracy, as well as the availability of sequence databases, are the most important requirements. New technologies in MS meet these prerequisites and have been successfully applied for primary sequence analysis and proteome profiling, posttranslational modifications, and protein–protein interaction analysis (Aebersold and Mann, 2003). Mass spectrometric identification of proteins is based on two independent approaches. The first is a combination of 2D gel electrophoresis and MS, whereas the second is a combination of protein purification steps, automated peptide MS/MS, and stable-isotope tagging of proteins and peptides for quantitation purposes. Three main approaches involving MS are currently used in proteomics: (1) peptide mass fingerprinting for protein identification, (2) sequence tags and postsource decay (PSD) analysis for protein or peptide sequencing, and (3) isotope coding for protein quantification.

For the typical mass spectrometric peptide mass fingerprinting, 2D gel electrophoresis is combined with MS. Therefore, the protein spots excised from the 2D gels are destained when necessary to ensure compatibility with the mass spectrometric application used. Then the spots are enzymatically digested to obtain smaller peptide fragments (Shevchenko et al., 1996). Most commonly, trypsin is the enzyme of choice, but chymotrypsin, formic acid, pepsin, inorganic cleaving agents, or combinations of these may also be used. A mass spectrometer consists of an ion source, by which the peptides are ionized, a mass analyzer, where ions are transmitted in an electromagnetic field, and an ion detector (Bakhtiar and Nelson, 2001; Aebersold and Mann, 2003). It is important to keep in mind that MS can only measure masses of molecules that can be ionized. For ionization, several methods can be used, including electron jet ionization (EI), chemical ionization (CI), fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-associated laser desorption/ionization (MALDI). The mass analyzer measures the mass-to-charge ratios (m/z) of the ionized peptides. The greater the accuracy of the measurement, the easier it is to identify the protein. Most common applications for mass analyzers include quadrupol, magnetic sector field, electric sector field, time-of-flight (TOF), electric ion trap, and electromagnetic ion trap (= electron cyclotron). The detector, which may be a Faraday's cup, a conversion dynode with secondary electron multiplier, a scintillation counter, or a multichannel plate, records the arrival of ions after they have passed through the mass analyzer.

In order to identify peptides and proteins, the resulting fragment mass spectra are submitted to online sequence databases, such as the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) or the SwissProt and TrEMBL databases at the ExPASy server (http://www.expasy.org) (*Figure 7.1-3*).

Table 7.1-3 lists several online tools available for the identification of specific mass spectra. These programs compare experimental spectra with theoretical spectra of the database entries by using distinct search algorithms (Sadygov et al., 2004).

Besides this peptide mapping or "fingerprinting" approach, MS can be used for protein or peptide sequencing in a PSD analysis (Jensen et al., 1999). PSD is based on ions that are generated in the ion source but are not stable (metastable ions). To obtain amino acid sequences, peptides are selected using electronic

Figure 7.1-3
Identification of proteins by MALDI-TOF MS and database search. Digested peptide ions are embedded in a matrix and immobilized on a metal surface. Peptides are ionized by a laser beam and accelerated in an electromagnetic field. The peptides reach the detector relative to their molecular mass and their charge. The experimental spectra are compared with theoretical spectra in sequence databases to find a matching amino acid sequence

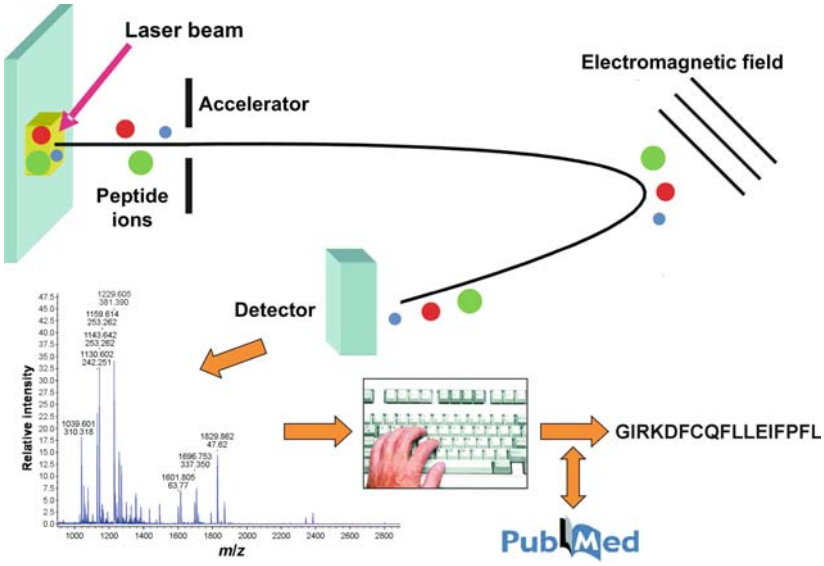


Table 7.1-3
Web-based analysis tools for data mining of peptide mass spectrograms. The measured fragment pattern is submitted to the Web search engine, which compares the fragments with in silico digested database entries

Application name	URL
Mascot	http://www.matrixscience.com
Masslynx	http://www.waters.com
MOWSE	http://www.hgmp.mrc.ac.uk/Bioinformatics/Webapp/mowse/
PeptIdent	http://www.expasy.org
PeptideSearch	http://www.narrador.embl-heidelberg.de/GroupPages/Homepage.html
ProbID	http://projects.systemsbiology.net/probid
ProFound v4.10.5	http://129.85.19.192/profound_bin/WebProFound.exe
ProteinProspector v4.0.5	http://prospector.ucsf.edu
PROWL	http://prowl.rockefeller.edu
SEQUEST	http://fields.scripps.edu, http://www.thermo.com
SpectrumMill	http://www.chem.agilent.com/
The Global Proteome Machine, X! Tandem	http://www.thegpm.org

pulses and their decay products are focused in a differential reflector field. More commonly, ESI is combined with triple–quadrupole or quadrupole/TOF hybrid analyzers. Therefore, the ions are selected in the first quadrupole and collide in the second one. Then their spectra are registered in the third one, or in

a reflector tube. The collision of peptides with gas molecules favors the fragmentation at the bound peptide, which allows easier identification of the spectra. In both cases, suitable software tools are necessary for database screening of the mass spectra.

The two "sequence tag" methods described can also be used in combination with other one- or multidimensional separation procedures such as chromatography (Mann et al., 2001). With this approach, proteins are not separated using 2D gel electrophoresis but are digested directly in the sample. The peptide solution is then separated by one of the various liquid chromatography (LC) methods available, and the eluted peptides are directly sequenced by mass spectroscopy. Using this approach, only a small amount of peptide (down to 1–2 molecules) is necessary for identification of the protein in the database. A different approach called "accurate mass and time (AMT) tag" combines the high resolution and mass measurement accuracy of Fourier transform ion cyclotron resonance (FITRC), the high efficiency of capillary LC separation, and the peptide identification capabilities of MS/MS (Pasa-Tolic et al., 2004).

2.2.2 Imaging MS

Although the described proteomic approaches allow the analysis of the protein components of a given sample, the regional distribution of individual "proteomes" cannot be determined easily. Imaging MS, developed by Stoeckli and Caprioli, allows a regional resolution of proteins present on a cryosection (Stoeckli et al., 2001). Brain samples are cut in a cryomicrotome and embedded on a gold surface. Then small pieces of the tissue are cut by laser beam of nearly half the size of a cell. The proteins and peptides present in the cut sample are then subjected to mass spectrometric identification. Then the laser beam moves on to the next point on the grid. Technical limitations allow an average distance of cutting points of about 100 mm, but technical improvement will decrease the grid size in the future to improve spatial resolution. The whole brain section can be scanned, producing several hundreds of data points, each of them consisting of several hundreds or thousands of mass spectra. Computer-aided reconstitution of the brain section can then show the regional distribution of a given protein in the whole section. Still the number of proteins isolated by laser dissection is small (about 100 per scan) and represents only a small range of proteins of the cell (some 10,000 per cell). Moreover, this method favors cytosolic proteins, and combination with other separation and identification techniques is difficult. On the other hand, it is intriguing to have a spatial resolution of proteins on a brain section.

2.2.3 Isotope Coding for Quantification Purposes

Stable isotope tagging has been used for quantitation and sequence identification of individual proteins within complex protein mixtures (Gygi et al., 1999a). The method is based on a new class of chemical reagents called isotope-coded affinity tags (ICAT) and tandem MS. In principle, two different biological samples are tagged with a "heavy" and a "light" isotope (e.g., deuterium and hydrogen), resulting in two protein samples with "light" or "heavy" isotope labels. The samples can be mixed and separated by LC. The corresponding heavy and light peptides will coelute from the chromatography column, and relative protein expression ratios can be measured in the subsequent MS by comparing the two peak intensities for the two isotopes. Furthermore, sequence information can be acquired for these peptides by fragment analysis in the product ion mass spectrum, as described above. ICAT requires the three functional elements of a specific chemical reactivity, an isotope-coded linker, and an affinity tag.

On the basis of this method, stable isotope labeling with amino acids in cell culture (SILAC) has been introduced for special application in cell culture (Ong et al., 2003). Cells of two biological conditions are cultured in amino acid-deficient growth media supplemented with radioactive amino acids labeled with two different isotopes (e.g., ^{12}C and ^{13}C). Differentially expressed proteins can be identified in the same way as in the ICAT method. SILAC uses global protein coding and does not require the functional chemical elements of ICAT.

Isotope coding and quantification of proteins requires that (1) all proteins/peptides in the sample are coded, (2) there is only a minimal spectral overlap between the isotopes used, (3) labeling is quantitative and the isotopic isoforms are enriched, (4) postcoding sample treatment does not influence mass spectrometric identification, (5) the resulting mass spectra are not influenced by the isotope coding, and (6) coding allows multiplexing two or more samples (Julka and Regnier, 2004). Several applications for isotope coding have been developed for expression analysis, posttranslational modifications, protein–protein interactions, single amino acid polymorphism, and absolute quantitation.

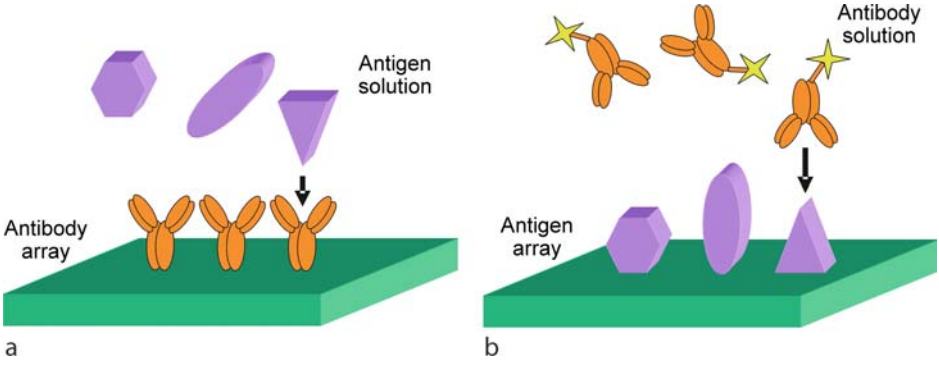
With regard to expression analysis, two different strategies are used (for review see Julka and Regnier, 2004). One is to label all peptides in a proteolytic digest of a proteome and then select specific peptides by chromatography in a second step. The other introduces isotope labels into peptides in a single-step reaction. General approaches include metabolic coding in isotopically enriched and depleted media, derivatization of primary amines, ^{18}O labeling, tagging amino and carboxyl groups at peptide termini (TACT), and esterification. Also, specific amino acids can be targeted by metabolic incorporation of amino acid-containing stable isotopes. Other ICAT reagents target cysteine, lysine, N-terminal threonine and serine, tryptophan, or methionine.

2.2.4 Protein Array Technology

As with gene chip microarrays, efforts have been undertaken to create protein-based microchips to analyze protein expression levels (James, 2002; Phizicky et al., 2003). The general principle is to immobilize protein molecules (e.g., antibodies) on a surface and to incubate with a protein sample solution (i.e., the antigens) for studying protein–protein interactions (*Figure 7.1-4*).

Figure 7.1-4

Protein array technology. In principle, two main approaches are used. (a) In the antigen capture array, different sets of antibodies are immobilized on a solid surface. For detection of the bound protein antigens, a secondary antibody system, or prelabeling of the proteins, is necessary. (b) In the direct assay, the protein antigens are immobilized and detected by labeled antibody probes (modified from MacBeath, 2002)



Three assay systems have been developed for protein microarrays (MacBeath, 2002):

1. Sandwich immunoassays—antibodies that bind to specific proteins in the sample are arrayed on a surface. If an antigen–antibody complex is formed, the complex is detected by a labeled secondary antibody.
2. Antigen capture immunoassays—proteins in the sample are labeled by specific molecular markers. These labeled proteins are bound by antibodies arrayed on a surface.
3. Direct immunoassays—antigens of the sample are attached to the chip surface and detected by labeled antibodies.

Because of new techniques in antibody engineering (Gavilondo and Larrick, 2000), the supply of antibodies as the limiting step in these approaches can be overcome. Other techniques using different protein recognition molecules such as phage-displayed antibodies, aptamers (short oligonucleotides with high protein affinity), or molecularly imprinted polymers (MIPs) still are in methodological development and are not ready for routine use (Jenkins and Pennington, 2001; Hanash, 2003).

Similar to other genomic and proteomic approaches, two main applications for protein arrays have emerged (MacBeath, 2002). First, the so-called unbiased, or discovery-orientated approach is used to separate, quantify, and identify as many proteins in a sample as possible. Often, two samples are compared to find differentially expressed proteins. This approach is similar to the 2D gel electrophoresis experiments. The second approach, the "focused", or "system-oriented" approach, gives a fast and less expensive way to analyze the characteristics of a large number of specified proteins. Only a subset of proteins is chosen by structural similarities due to their sequence or functional relationship, and analyzed. Microarray technology is particularly well suited for the second approach, although the proteins to be studied have to been known and well characterized.

Although gene chip and protein chip experiments share some common principles, several limitations have to be taken into account. The complexity of the proteome exceeds the complexity of the genome by decimal power. For example, the known 30,000–40,000 human genes will be translated approximately into more than 100,000–150,000 proteins. This makes it difficult to construct miniature "whole proteome" chips. Moreover, no function is known for about 75% of the proteins, which limits their diagnostic use in clinical application. It also has to be taken into account that the dynamic range of protein expression can exceed 10^7 (Kusnezow and Hoheisel, 2002), making it difficult to construct detection systems that can be applied for the whole range of expression and still show sufficient information to find differential expression (Gygi et al., 2000a).

Besides these general considerations, protein array technology has also several technical limitations compared with gene chip arrays. First, nucleic acids can be amplified by generally established techniques such as polymerase chain reaction (PCR), whereas such methods for amplification of proteins do not exist, making it difficult to provide a sufficient number of molecules for large-scale applications; second, proteins are more complex and heterogeneous than nucleic acids with regard to structure and function, which prevents a more detailed analysis of their characteristics rather than their presence in most cases; third, proteins easily loose their biological activity due to chemical modification or denaturation and therefore proteins cannot be as easily attached to chip surfaces as nucleic acids and stored for some time without loosing biological activity; fourth, antibody–antigen interactions are varying in their specificity and affinity, resulting in diversities of protein binding (Jenkins and Pennington, 2001; Kusnezow and Hoheisel, 2002; Valle and Jendoubi, 2003).

One of the inherent problems of proteomics based on 2D gels is the poor resolution of membrane-bound proteins. Therefore, most of the proteins located on the cell surface are not included in 2D gels. Iwato and coworkers have recently demonstrated an elegant method for screening cell surface markers using an antibody array (Ko et al., 2005a, b). The arrays consisted of antibodies bound to cellulose membranes or glass plates. This approach made it possible to expose whole cells to the microarrays and to detect cell surface proteins by immunocytochemistry. In the future, further miniaturization and the use of additional antibodies will allow refining this attractive method in cell biology.

SELDI-TOF. Another application utilizing affinity surfaces to retain proteins based on their physical and chemical characteristics combined with direct TOF-MS is surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (Merchant and Weinberger, 2000). Protein retention is performed by chromatography with varying properties such as anion exchange, cation exchange, metal affinity, and reverse phase. By applying differing chip conditions in parallel or in series, complex biological protein mixtures can be resolved into subsets of proteins with common properties (Fung and Enderwick, 2002). The main advantage of SELDI-TOF-MS technology is in its rapid screening capabilities, which are useful for fast clinical detection of differential protein expression, e.g., in cancer and infectious disease medicine.

2.3 Bioinformatics

Though the whole field of proteomics emerged only after major technological advances, the biggest steps have been undertaken in computer technology (Chakravarti et al., 2002). Proteome informatics involves not only collection, storage, search, analysis, and classification of experimental results, but also integration, management, and retrieval of data from large-scale databases. Thus, whole proteomic technology is mainly based on computer analysis. Two-dimensional gel electrophoresis software, automated spot picking, sequence database search, proteome databases, and protein interaction maps are all dependent on powerful bioinformatic tools to translate experimental results into meaningful biological answers (Dowsey et al., 2003).

2.3.1 Web Databases

Internet databases are a common application to share proteomic data with a large number of other scientists. Because of the large size of data and a dynamic turnover of protein information including functional annotation, it is not possible in print publications any more. Numerous publications describing proteomic profiles of a large number of tissues or organisms made it necessary to develop rules for federated 2D databases (Appel et al., 1996, 1999). These rules comprehend:

1. Keyword search—Individual entries in the database must be searchable and accessible by remote keyword search.
2. Hypertext links—The database must be linked to other databases through active hypertext cross-referencing.
3. Main index—All databases of a site must have a single entry point.

An incomplete list of currently existing Internet databases can be found in *Table 7.1-4*. We have checked the hyperlinks carefully, but these databases are in current flow, as they are constantly growing and modified.

2.3.2 Online Tools

Indexing proteomes on the Web is only a part of proteomic data management. It is necessary to apply computer-aided analysis to retrieve useful information from the proteome data and to compare results from different experimental approaches.

The largest set of tools collected from the Swiss Institute of Bioinformatics can be found at the ExPASy (Expert Protein Analysis System) proteomics server at the URL http://www.expasy.org, including software that can be used to identify mass spectrometric data, to translate DNA into protein sequences, to search for sequence similarities, to search for protein sequence patterns, to predict posttranslational modifications and topology, to analyze the protein primary, secondary, or tertiary structure, and to align sequences (Hoogland et al., 1999; Wilkins et al., 1999).

Other resources are available:

- Gelscape (http://www.gelscape.ualberta.ca), an automated system for comparison of gel images (Young et al., 2004);
- Matchminer (http://discover.nci.nih.gov/matchminer), a tool to navigate among gene and gene product identifiers (Bussey et al., 2003);
- GoMiner (http://discover.nci.nih.gov/gominer), a tool for biological interpretation of genomic and proteomic data (Zeeberg et al., 2003);
- Cluster and Treeview (http://rana.lbl.gov), for cluster analysis of genomic and proteomic data (Eisen et al., 1998);
- Expression Profiler (http://ep.ebi.ac.uk/EP), a set of programs for the analysis of genomic and proteomic data sets (Vilo et al., 2003);

Table 7.1-4

Two-dimensional databases available on the Internet. Some of the databases are specialized in one specific tissue or cell line, whereas others comprehend a global list with links to other databases. Most databases contain proteins that have been isolated from the brain or cerebrospinal fluid and are therefore useful in brain research. (Enlarged and updated table modified from Lopez, 1999)

Name	URL	Description
2-D PAGE Aberdeen	http://www.abdn.ac.uk/~mmb023/2dhome.htm	*Haemophilus influenzae* and *Neisseria meningitidis*
Argonne Protein Mapping Group	http://www.anl.gov/BIO/PMG/	Mouse liver, human breast cell lines, pyrococcus
BALF 2D_AGE	http://www.umh.ac.be/~biochim/BALF2D.html	Mouse, human bronchoalveolar lavage fluid
Danish Centre for Human Genome Research	http://biobase.dk/cgi-bin/celis/	Human: primary keratinocytes, epithelial, hematopoietic, mesenchymal, tumors, urothelium, amnion fluid, serum, urine, proteasomes, ribosomes, phosphorylations. Mouse: epithelial, new born (ear, heart, liver, lung)
ECO2DBASE (in NCBI repository)	ftp://ncbi.nlm.nih.gov/repository/ECO2DBASE/	*Escherichia coli*
ExPASy SWISS-2DPAGE	http://www.expasy.ch/	Liver, plasma, HepG2, HepG2SP, RBC, lymphoma, CSF, macrophage-CL, erythroleukemia-CL, platelet, yeast, *E. coli*, colorectal, kidney, muscle, macrophage-like-CL, pancreatic islets, epididymus, dictyostelium
GelBank-Argonne National Lab	http://gelbank.anl.gov/overview/	42 bacterial genomes
Heart High-Performance 2-DE Database	http://www.mdc-berlin.de/~emu/heart/heart.html	Human heart
Heart-2D PAGE, The Human Myocardial Two-Dimensional (2D) Electrophoresis Protein Database	http://www.chemie.fu-berlin.de/user/pleiss/	Human heart
HSC-2D PAGE, Heart Science Centre, Harefield Hospital	http://www.harefield.nthames.nhs.uk/	Human, rat and mouse heart
Immunobiology, University of Edinburgh	http://www.ed.ac.uk/~nh/2DPAGE.html	Embryonal stem cells
INRA Maize Genome Database	http://moulon.moulon.inra.fr/imgd	Maize
IPS/LECB, NCI/FCRDC	http://www.lecb.ncifcrf.gov/ips-databases.html	Phosphoprotein, prostate, breast cancer drug screen, FAS (plasma), Cd toxicity (urine), leukemia
Joint Protein Structure Lab	http://www.ludwig.edu.au/jpsl/jpslhome.html	Human colorectal-CL, placental lysosomes
Lab. de Biochimie et Tech. des Proteines, Bobigny	http://www-smbh.univ-paris13.fr/lbtp/biochemistry/biochimie/bque.htm	Human leukemia cell lines
Large Scale Biology Corp	http://www.lsbc.com/	Rat, mouse, human liver, corn, wheat
Max-Planck-Institut f. Infektionsbiologie	http://www.mpiib-berlin.mpg.de/2D-PAGE	*Mycobacterium tuberculosis*, vaccine strain *Mycobacterium bovis* BCG

Table 7.1-4 (Continued)

Name	URL	Description
Meta-Database Catalog of 2D gel images found in Web databases – 2DWG	http://www-lmmb.ncifcrf.gov/2dwgDB/2DWG.html	Set of links to www sites
Mito-pick	http://www-dsv.cea.fr/thema/MitoPick/Mito2D.html	Human mitochondria
Molecular Anatomy Laboratory	http://iupucbio1.iupui.edu/Frankw/molan.htm	Rat
Mouse Proteome Database	http://www.ukrv.de/humangenetik	Mouse brain
Partial List of Web 2D Electrophoretic Gel Databases	http://www.lecb.ncifcrf.gov/EP/table2Ddatabases.html	Set of links to 2D gel Web databases
PDD (Protein Disease Database)	http://www-lecb.ncifcrf.gov/PDD	Plasma, cerebrospinal spinal fluid, urine
PHCI-2DPAGE	http://www.gram.au.dk/	Parasite–host cell interaction, IFN-γ-induced HeLa cells
PMMA-2DPAGE	http://www.pmma.pmfhk.cz/	Human colorectal carcinoma
PPDB (Phosphoprotein Database)	http://www-lecb.ncifcrf.gov/phosphoDB/	Phosphoproteins: murine B lymphoma cells (WEHI-231)
Protein Project of Cyanobacteria	http://www.kazusa.or.jp/cyano/cyano2D/	Cyano2Dbase-*Synechocystis* sp. PCC6803
PROTEOME Inc (YPD-Yeast Protein DB)	http://www.proteome.com/	Yeast
Siena2D-PAGE	http://www.bio-mol.unisi.it/2d/2d.html	*Chlamydia trachomatis* L2, *Caenorhabditis elegans*, Human breast ductal carcinoma and histologically normal tissue, human amniotic fluid
ToothPrint DB	http://bioc111.otago.ac.nz:8001/tooth/home.htm	Dental tissue in rat
UCSF 2D PAGE	http://rafael.ucsf.edu/2DPAGEhome.html	A375 melanoma cell line
University of Greifswald, Germany	http://pc13mi.biologie.uni-greifswald.de/	*Bacillus subtilis*
Washington University Inner Ear Protein Database	http://oto.wustl.edu/thc/innerear2d.htm	Humans: inner ear
Yeast 2D gel DB, Bordeaux	http://www.ibgc.u-bordeaux2.fr/YPM	Yeast
YPD (Yeast Protein Database)	http://www.proteome.com/YPDhome.html	Yeast

- JVirGel (http://prodoric.tu-bs.de/proteomics.php), a tool for the construction of virtual gel images, which allows the identification of unknown proteins and the localization of known proteins on gel images (Hiller et al., 2003); and
- GenMAPP (http://www.genemapp.org), which allows one to display and modify own results from gene expression experiments in pathway diagrams (Dahlquist et al., 2002; Doniger et al., 2003).

We have collected a list of gene and protein expression analysis tools (Maurer, 2004a) including databases, structure prediction websites, and other data interpretation Internet pages and programs, with the emphasis on programs and sites freely available to the research community.

2.3.3 Statistical Data Analysis

One important aspect to consider is that the statistical approach used for the analysis of 2DE experiments has a major impact on the outcome of the experiment. Therefore, care must be taken to choose the appropriate statistical method.

Recently, we have compared three different statistical approaches applied to the same data set (Maurer et al., 2005). We found major differences in the results calculated by *t*-test statistics and the statistical algorithms included in the 2D software package used. Additionally, applying software for cDNA/oligonucleotide microarray experiment analysis and comparing the results to the 2D statistics showed that care should also be taken when transferring the microarray statistical algorithms to proteome data sets. Microarrays normally consist of several thousands of sequences, whereas 2D proteomic experiments identify some hundred proteins at their best, meaning that there is at least one order of magnitude between genomic and proteomic items that needs to be compared statistically. It seems that microarray software does not produce very stringent results in proteomic experiments due to the relatively small data sets in 2D analysis.

In a recent review (Boguski and McIntosh, 2003), the authors discuss statistical methods used for the identification of peptides in proteomic experiments based on MS. They compare the bioinformatics scoring systems for detecting false-positive or false-negative sequences in MS to the false detection rate (FDR) of microarray experiments. To summarize their considerations, it is important to keep in mind that for large-scale experiments such as genomics and proteomics large number of genes or proteins are misidentified just by chance. In consequence, the statistical analysis had to be more stringent or a new statistical methodology needs to be developed to address these questions. Unfortunately, there is no reliable way to deal with these issues currently.

3 Proteomics and the Analysis of Cerebral Energy Metabolism

Proteomic technology can be useful for the analysis of proteins with known enzymatic functions in cerebral cellular metabolism. Since the components of the basic metabolic pathways are well described on both the structural and the functional level, it is possible to identify the proteins involved. Using proteomic technology, the advantage to analyze a large number of these proteins at once is obvious.

3.1 Enzyme Function and 2D Gel Electrophoresis

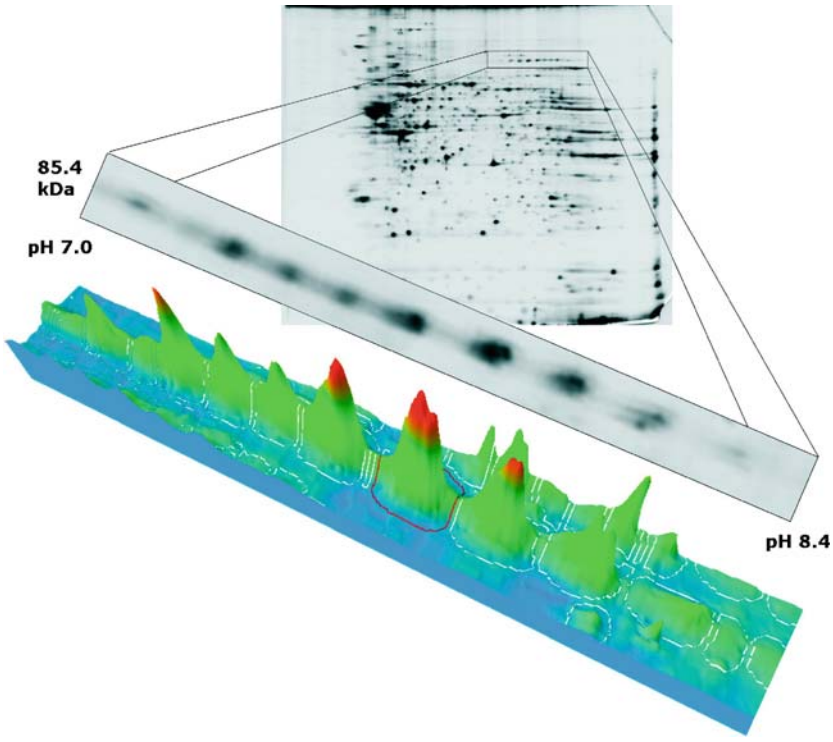
A question that needs to be addressed is whether the protein content represented by the spot volumes on 2D gels correlates with the enzymatic function of the respective protein. In an early study, Merril and Goldman found that the amount of protein in a protein spot on 2D gels correlates well with the enzymatic function of the protein investigated (Merril and Goldman, 1982). Since such findings may not hold for every experimental condition, care should be taken correlating protein concentration with protein function. Thus, proteins may be inactive, or they may become inactivated. In addition, 2D gel electrophoresis may result in separate isoforms, which may exert different functional effects such as different catalytic capacities that are not related to the spot volumes (*Figure 7.1-5*). In contrast, most enzymatic tests do not separate different enzyme subtypes or isotypes, but they determine an overall enzymatic function.

3.2 Glucose Metabolism and Cellular Respiration

Proteomics based on 2D gel electrophoresis is an extremely useful tool for investigating metabolic pathways, because the majority of proteins in these pathways is located in the cytoplasm and 2DE is very well suited to separate predominantly cytoplasmic proteins due to their mostly hydrophilic properties, isoelectric points around pH 6–7, and a molecular mass up to 200 kDa.

Figure 7.1-5

Proteomics used for the identification of protein isoforms. In a two-dimensional (2D) gel experiment (Maurer et al., 2003b), we identified about 12 protein isoforms of the mitochondrial aconitase (ACO2). These isoforms are mainly generated by posttranslational modifications such as phosphorylation. A theoretical search for serine, threonine, and tyrosine phosphorylation sites at http://www.cbs.dtu.dk revealed a total of 30 possible sites. This means that, theoretically, the same number of protein isoform spots can be present on a 2D gel. The ACO2 enzyme plays a role in energy metabolism and iron homeostasis and is related to Friedreich's ataxia

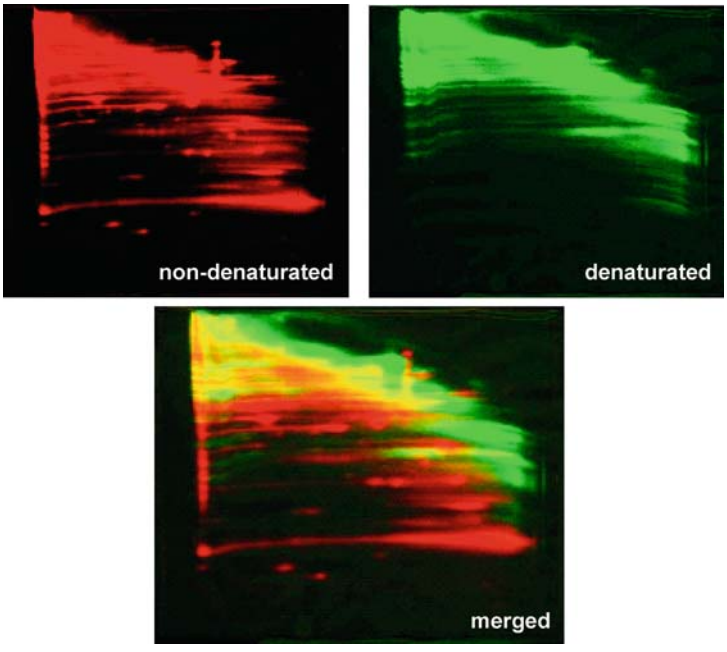


It is possible to evaluate the composition of individual components of the glycolytic pathway simultaneously. For example, different isoforms (including posttranslationally modified proteins) can be observed and their enzymatic function visualized by specific staining procedures. Of note, standard 2DE is a denaturating gel electrophoresis. This means that the protein content can be measured easily; however, for a functional analysis, other types of extraction protocols and nondenaturating gel electrophoresis have to be applied to maintain proteins in their functional state. For in-gel staining of enzymatic function, Rothe (1994) has compiled a huge collection of protocols that are an invaluable tool for subsequent functional analysis.

As a method of nondenaturating gel electrophoresis, blue-native 2D gel electrophoresis has been developed, which allows to separate intact proteins or protein complexes (Schägger and von Jagow, 1991; Schägger et al., 1994; Camacho-Carvajal et al., 2004). In contrast to standard 2DE, the first dimension is not a protein separation according to the protein isoelectric point, but rather a molecular mass separation. Therefore, the protein patterns occurring on the 2D gels are different to the gel images seen using denaturating 2DE. The blue-native 2DE is extremely useful for finding protein–protein interactions or to identify multiprotein complexes (*Figure 7.1-6*).

Metabolic pathway analysis allows the monitoring of a large number of proteins simultaneously. Ideally, all proteins of an enzymatic pathway can be identified on the 2D gels, although only few studies aimed at

Figure 7.1-6
Identification of multiprotein complexes using blue-native two-dimensional (2D) gel electrophoresis. We separated proteins isolated from whole-cell lysates of hippocampal neural stem cells. *Upper panel*: In the first dimension, proteins were separated under nondenaturating (*left*) or denaturating (*right*) conditions, followed by a denaturating separation in the second dimension. *Lower panel*: The merged image shows monomeric proteins, multimeric proteins (*dark gray*), and the monomers of which the multimeric proteins are consisting of (*light gray*) (Maurer et al., unpublished data)



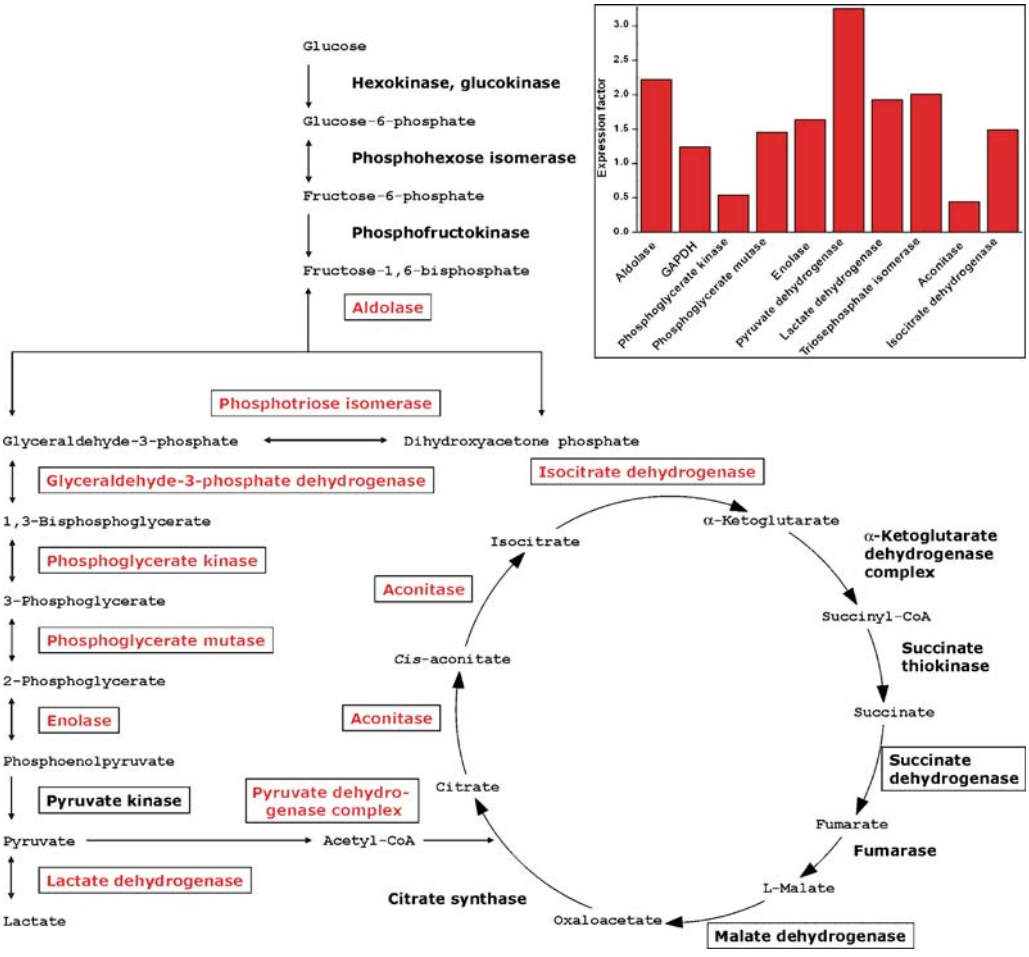
creating pathway maps for metabolic pathways. One explanation for this remarkable neglect is that the pathways are well defined, such as those of glycolysis or of the Krebs cycle. Their description in the biochemistry textbooks may leave one with the impression that these proteins are uninteresting targets for screening studies, which are always aimed at searching for new and unknown protein targets. For example, the metabolically well-known glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), termed the "housekeeping protein" with all its connotation, reemerged as a protein with a vast variety of other functions, including the control of transcription and translation, apoptosis, and membrane fusion (Sirover, 1999).

To set a paradigm for metabolic pathway analysis, we reevaluated a proteomic study of our own to analyze proteins in metabolic pathways. We separated proteins from undifferentiated hippocampal neural stem cells by 2DE and compared the results to the protein pattern of in vitro differentiated cells (Maurer et al., 2004). With regard to enzymes involved in glucose metabolism, we identified nearly all of the proteins involved in glycolysis and the Krebs cycle (*Figure 7.1-7*). Most of the identified proteins were differentially expressed under in vitro differentiating conditions, indicating that the cellular metabolism undergoes major changes and adapts to the new needs of the differentiated cell.

Metabolic pathway analysis in cell culture models can also be used for monitoring the effects of nutrient supply and demand. Several studies investigated the influence of different feeding conditions on cell growth and proliferation, e.g., by varying the concentration of glucose. It was possible to correlate the intracellular metabolic changes with the physiological metabolic shift in lactate and ammonia metabolism (Seow et al., 2001; Korke et al., 2004).

Figure 7.1-7

Proteomics used for metabolic pathway analysis. Proteomic technology allows monitoring a large number of proteins simultaneously. In this example, we compared changes in the proteomes of undifferentiated neural stem cells with in vitro differentiated cells (Maurer et al., 2004). We could identify a large number of enzymes involved in glycolysis and the Krebs cycle (*black boxes*), most of them showing differential protein expression upon differentiation (*italics*). *Inset*: Quantitative changes between undifferentiated and in vitro differentiated neural stem cells. The columns show the expression factor, defined as the differentiated/undifferentiated ratio



In nearly all studies involved in creating brain proteome databases (for a list of selected studies, see *Table 7.1-5*), the group of enzyme proteins involved in intermediary metabolism is represented most dominantly not only by number, but also as highly abundant proteins (Lubec et al., 2003). This allows pathway analysis of enzyme activity and can be used for monitoring the inborn errors of metabolism in the brain.

Proteomic methodology can also be used for chemotyping of enzyme classes. Enzymes have common structural properties defining their function. In a recent study, the authors used a combinatorial strategy to identify at least six mechanistically distinct enzyme classes of sulfonate ester-containing proteins (Adam et al., 2002).

Table 7.1-5
Brain proteome studies[a]

Brain proteome databases	
Mouse brain	Eckerskorn et al. (1988), Jungblut et al. (1992), Gauss et al. (1999), Klose (1999), Tsugita et al. (2000), Klose et al. (2002)
Rat brain	Fountoulakis et al. (1999, 2000)
Human brain	Karlsson et al. (1999), Langen et al. (1999)
Human frontal cortex	Lubec et al. (2003)
Human hippocampus	Yang et al. (2004)
Human brain (HUPO, planned)	Marcus et al. (2004)
Experimental disease models	
Focal cerebral ischemia	Jacewicz et al. (1986), Kiessling et al. (1986)
Excitotoxicity	Krapfenbauer et al. (2001)
p53 knockout mouse brain (tumor suppressor gene involved in neuronal apoptosis)	Araki et al. (2000)
E1 mouse brain (epileptic seizures)	Ohmori et al. (1999)
GSK3β transgenic mouse (Alzheimer's disease)	Tilleman et al. (2002)
Cell culture	
Neuronal hypoxia	Jin et al. (2004)
Adult rat hippocampal neuronal stem cells	Maurer et al. (2003b, 2004)
Human fetal brain stem cells	Pearce and Svendsen (1999)
Murine embryonic stem cells	Guo et al. (2001)
Postnatal rat cerebellum	Taoka et al. (2000)
Human pluripotent stem cells	Hayman and Przyborski (2004)
Human CSF and disease models	
CSF in schizophrenia, affective disorders, multiple sclerosis, neurosyphilis, AIDS, Alzheimer's disease, Parkinson's disease, infectious diseases of the brain, epilepsy, amyotrophic lateral sclerosis, polyneuropathy, and many others	Goldman et al. (1980), Harrington and Merril (1984, 1988), Harrington et al. (1984), Walsh et al. (1984), Bracco et al. (1985), Wiederkehr et al. (1985), Pirttila et al. (1991), Wildenauer et al. (1991)
Human CSF reference map	Sanchez et al. (1995), Sickmann et al. (2000, 2002)
CSF in Alzheimer's disease	Davidsson et al. (2001, 2002), Puchades et al. (2003)
CSF in traumatic brain injury	Conti et al. (2004)
CSF in neurologically normal, elderly patients	Wenner et al. (2004)
CSF in lower back pain patients	Yuan et al. (2002)
Human brain disease	
Alzheimer's disease	Lubec et al. (1999), Castegna et al. (2002a, b, 2003), Kim et al. (2002, 2004), Tsuji et al. (2002), Butterfield et al. (2003)
Down's syndrome (trisomy 21)	Bajo et al. (2002), Jae-Kyung et al. (2003), Shin et al. (2004a)
Human brain microdialysate, stroke	Maurer et al. (2003a)

[a]This table includes a representative collection of brain proteome studies. A huge number of other brain proteomic studies could not be included due to space constraints

Rabilloud et al. (2002) investigated changes in the cellular proteome after oxidative stress. They focused on the cellular reaction to peroxiredoxins, enzymes destructing reactive oxygen species (ROS) such as peroxides. Oxidative stress also seems to play a major role in neurodegenerative diseases such as Alzheimer's disease (Castegna et al., 2002a, b; Butterfield, 2004) (see below).

3.3 Mitochondrial Proteomics

The analysis of mitochondrial proteins under normal and disease conditions has assumed importance. Mitochondria play an essential role in cellular metabolism by providing cellular energy through ATP synthesis. The enzymes for oxidative phosphorylation are located in mitochondria, as well as some of the enzymes necessary for free fatty acid metabolism and the Krebs cycle. Other biochemical pathways in mitochondria involve heme biosynthesis, ketone body generation, and hormone synthesis. Mitochondria also play a major role in the generation and degradation of ROS. Moreover, they are involved in key pathways in cellular signaling such as calcium signaling and apoptosis.

Subcellular isolation techniques enable the separation of the mitochondrial compartment of the cell, which can be analyzed in proteomic studies. Prerequisite for a functional proteomic analysis of mitochondria in disease models is the knowledge of the proteins that are normally present in mitochondria (Westermann and Neupert, 2003). A study based on liquid chromatography tandem mass spectrometry (LC-MS/MS) provided a comprehensive proteomic database of 591 proteins found in mitochondria isolated from mouse brain, heart, kidney, and liver (Mootha et al., 2003), whereas a study based on minispin affinity columns generated profiles of calcium-binding proteins, glycoproteins, and membrane proteins in the mitochondrial fraction (Lopez et al., 2000). A study using 2D gel electrophoresis provided a reference map for mitochondrial proteins (Krapfenbauer et al., 2003).

Even though mitochondria perform a multitude of different functions in cellular physiology, it is of interest to know the contribution of altered mitochondrial function to disease processes like neurodegeneration, obesity, cancer, diabetes, aging, and cardiomyopathy (Wallace, 1999). Mitochondrial proteomics may play a role in diseases where mitochondrial dysfunction has been implicated, such as in neoplastic growth (cancer), neurodegeneration (Alzheimer's disease), or metabolic disorders (diabetes). The knowledge of the mitochondrial proteome and the changes therein due to pathophysiological events will be useful for drug testing and diagnostic improvements.

3.4 Proteomic Studies Investigating Brain Metabolism in Brain Disease or Disease Models

3.4.1 Inborn Errors of Metabolism

Early studies investigated inborn errors of metabolism by means of 2D gel electrophoresis (Merril and Goldman, 1982). The authors found alterations of proteins in lymphocytes of patients with Lesch–Nyhan syndrome, a neuropsychiatric disease that is associated with self-mutilation, spasticity, and hyperuricemia. The patients have decreased or absent levels of the purine-modifying enzyme hypoxanthine phosphoribosyltransferase (EC 2.4.2.8). Although the disease is extremely uncommon, it was one of the first neuropsychiatric diseases of which the molecular mechanisms were elucidated. The authors found 17 protein spots with differential expression; unfortunately, none of them could be identified, since MS was not developed for proteomic analysis at that time.

3.4.2 Down's Syndrome (Trisomy 21)

Down's syndrome (trisomy 21) is a common genetic cause of human mental retardation. In patients suffering from Down's syndrome, deteriorated glucose, lipid, purine, folate, and methionine/homocysteine metabolism have been reported. Lubec and Fountoulakis and coworkers have investigated proteomic changes in fetal brain tissue from patients with Down's syndrome. They found a derangement of intermediary metabolism during prenatal development, with increased protein levels of mitochondrial aconitase, NADP-linked isocitrate dehydrogenase, and pyruvate kinase as well as decreased protein expression of citrate synthase, cytosolic aspartate aminotransferase, and hypothetical proteins 4833418L03Rik protein Q9D614, mitochondrial inner-membrane protein Q16891, Nit protein 2 Q8WUF0, KIAA1185,

hypothetical protein 55.2 kDa, hypothetical protein 58.8 kDa, actin-related protein 3β (ARP3beta), and putative GTP-binding protein PTD004 (Bajo et al., 2002; Jae-Kyung et al., 2003; Shin et al., 2004a). Also in animal models of Down's syndrome, like a transgenic mouse overexpressing human Cu/Zn superoxide dismutase I (SOD1), hypothetical hippocampal proteins of 2610008O03Rik protein Q9D0K2 and 4632432E04Rik protein Q9D358 were decreased and hypothetical protein Q99KP6 was increased, indicating an impairment of metabolism, signaling, and transcription machinery in the SOD1 transgenic brain (Shin et al., 2004b). Other studies investigating brain proteomes of transgenic mice can be found in *Table 7.1-5*.

3.4.3 Alzheimer's Disease

Neurodegenerative disorders have been in the focus of proteomic analysis, mostly as their pathobiological events can be seen at the protein level. Neurodegenerative diseases are characterized by the loss of neurons and the intracerebral accumulation of fibrillary material. Some of the pathophysiological mechanisms have been elucidated and include (1) alterations in calcium homeostasis in the endoplasmic reticulum resulting in excitotoxicity, proteolysis, and neuronal apoptosis, (2) mitochondrial dysfunction resulting in free radical generation and impaired calcium buffering, and (3) disorganization of the cytoskeleton resulting in neurodegeneration (Tsuji et al., 2002).

Alzheimer's disease is one of the most common causes of dementia in the elderly, affecting more than 10% of the population over 65 years of age. The neuropathology of Alzheimer's disease involves irreversible progression of dementia, neuronal cell death, and accumulated protein aggregates consisting of amyloid and tau protein. Mutations that are responsible for familial Alzheimer's disease in the amyloid β precursor protein (APP) and presenilin-1 and -2 have been identified. Also, the microtubule-associated protein tau is abnormally phosphorylated, which prevents its degradation by the ubiquitin-proteasome system, resulting in an aggregation to protein complexes, the so-called tangles (Kim et al., 2004).

Studies investigating Alzheimer's disease focused mainly on two biochemical processes: (1) the formation of the neurotoxic Aβ peptide in senile plaques by a sequence of posttranslational modifications of the amyloid β precursor protein, and (2) the hyperphosphorylation of tau protein as the major component of tangles (Kim et al., 2004). As both pathways are not reflected by genomic alterations, several studies have applied proteomics for detecting changes in protein expression in the brains of patients with Alzheimer's disease. The main proteome alterations detected in such brains involve synaptic loss, oxidative stress, decreased glucose metabolism and protein turnover, mitochondrial dysfunction, and deficits in protein folding (Lubec et al., 1999; Castegna et al., 2002a, b, 2003; Kim et al., 2002, 2004; Tsuji et al., 2002; Butterfield et al., 2003).

Several glycolytic enzymes including α- and γ-enolase (phosphopyruvate hydratase) and glyceraldehyde-3-phosphate dehydrogenase show decreased expression in the brains of patients with Alzheimer's disease. Moreover, the expression of voltage-dependent anion channel-1 (VDAC-1) is decreased. This protein regulates the mitochondrial metabolism by controlling the ATP flux. The expression of the complex I of the mitochondrial electron transport chain (synonymously NADH:ubiquinone oxidoreductase) was also reported to be decreased, indicating that ATP production might be impaired (Butterfield, 2004). Other findings involve a decreased expression of creatine kinase (BB isoform) and triosephosphate isomerase, which are also involved in ATP production. The dysregulated energy supply may affect chemical gradients, ion channels, and finally the cell potential, resulting in neurodegeneration (Butterfield et al., 2003).

Contrary to the findings in other studies investigating the cerebrospinal fluid proteome from patients with Alzheimer's disease, the metabolic proteins could not be detected in the cerebrospinal fluid in this study, indicating that the pathological processes remain located intracellularly. The observed alterations in apoliprotein E (ApoE) and its precursor proapolipoprotein, as well as in transthyretin, β-2 microglobulin, retinol-binding protein, and Zn-α-2 glycoprotein, may be explained by the involvement of βAPP in other mechanisms like cholesterol metabolism (Davidsson et al., 2002, 2003).

A mouse model for Alzheimer's disease is the GSK3β transgenic mouse, which overexpresses the multifunctional enzyme glycogen synthase kinase-3β (GSK3β). GSK3β phosphorylates the tau protein, which then aggregates. These aggregates seem to play a pathogenetic role in Alzheimer's disease, as in the transgenic mouse, hyperphosphorylation of the tau protein is followed by increased formation of neurofibrillary tangles. Tilleman et al. (2002) used 2D gel electrophoresis to find 51 differentially expressed protein spots in the transgenic mice as compared with wild-type littermates. They identified downstream targets of the GSK3β signaling pathways in the functional groups relating to cytoskeleton, energy metabolism, folding, signal transduction, detoxification, and oxygen scavenging.

In summary, the numerous studies investigating the proteome of diseased brain tissue or cerebrospinal fluid could not discriminate distinctively and unanimously between cause and effect of their observations. Although the metabolic changes of brain metabolism after a noxious event can be monitored, the meaning of "marker" proteins found for various diseases cannot be determined easily. Similar to the results observed in genetic testing, the observation of a specific protein in a specific condition is not necessarily related in a pathophysiological sequence of events, and its clinical relevance remains unclear. Thus, all candidate markers have to be carefully evaluated further, both in other experimental approaches in vitro and in vivo as well as in clinical trials.

4 Perspectives

4.1 Outlook: Challenges and Issues To Be Resolved

4.1.1 General Considerations

Application of proteomic techniques has inherent problems of biological and technical nature. Biological limitations concern interindividual diversity of biological samples, resulting in a large scatter of data. In addition, protein concentrations may be altered by degradation after sampling. Furthermore, results may be altered by posttranslational modifications or the influence of drugs. Finally, protein expression changes over time due to different environmental cues in development and aging, making it necessary to investigate different time points.

Technical limitations result from the fact that only a finite number of the most abundant proteins can be resolved and stained on the 2D gels, but the dynamic range of protein expression exceeds a factor of 10^7 (Tyers and Mann, 2003). The process of gel analysis is time-consuming and labor-intensive. Finally, MS requires expensive equipment as well as specially trained personnel. Future developments will be based on technological advances like miniaturization and increased automatization of instrumentation as well as further acceleration in high-throughput screening.

4.1.2 Membrane Proteins

Biological membranes are important in the processing of information, which is of special relevance for the brain. Integrated membrane proteins such as ion channels, receptors, and transporters together with cytoskeletal elements contribute to neural and astrocytic signaling. Both neurogenesis and gliogenesis require a constant flow of membranes as the basis of morphological changes. Finally, cellular metabolism in the brain is dependent on intact membranes and on integral membrane proteins that mediate the flux of metabolites.

Although it is desirable to detect membrane proteins by proteomics, membrane proteins are underrepresented in 2D gels. This is due to the following: (1) They are less abundant proteins, which makes them "invisible" in the pool of cytoplasmic proteins on standard 2D gels; (2) they have mostly alkaline isoelectric points (pI $>$ 8), which would require technically more difficult alkaline pH gradient gels; and (3) most of their domains are composed of hydrophobic amino acids, meaning that these proteins are rarely soluble in

the standard aqueous buffer systems used for first-dimensional IEF in 2D gel electrophoresis. Membrane proteins tend to precipitate during this step (Santoni et al., 2000). Although promising improvements including differential solubilization techniques and new surfactants and detergents for membrane protein extraction have been described (Santoni et al., 2000), electrophoresis-free methods such as direct MS have been developed (Aebersold and Mann, 2003), or alternatively, electrophoresis under nondenaturating conditions such as the blue-native 2D electrophoresis has been applied for resolution of membrane proteins (Schägger et al., 1994; Brookes et al., 2002; Camacho-Carvajal et al., 2004). Future research may also concentrate on the development of detergents to solubilize integral membrane proteins compatible with IEF, which would then allow 2D gel electrophoresis for membrane proteins.

A different approach for the analysis of membrane proteins is called multidimensional protein identification technology (MudPIT) (Washburn et al., 2001). It combines a series of prefractionation steps including membrane solubilization, enzymatic digestion of the proteins, strong cationic exchange columns (SCX technology), and reverse-phase high-pressure liquid chromatography (RP-HPLC), with the advantages of electrospray MS (ESI-MS/MS). This technique allows the rapid identification of integral membrane proteins, but it is not quantitative.

4.1.3 Dynamics of Protein Turnover

The cellular protein concentration is balanced in a constant flow from synthesis to degradation (steady state). As the current proteomic technology can only display a small section of the protein content at a given time point, rates of protein turnover need to be determined separately to get an impression of the cellular protein turnover. Pratt et al. (2002) used stable isotope-labeled amino acids to monitor mass shifts in tryptic protein fragments. This approach allows determining rates of protein turnover and protein half-lives by MS. Such an approach is easier and more comprehensive than traditional methods such as liquid scintillation or autoradiography.

4.1.4 Quantitation

In most proteomic studies, two or more groups are compared on a qualitative basis: The presence or absence of a spot is detected in one of the experimental groups. It is more difficult to obtain quantitative data, since the technique of analysis is limited: protein isolation protocols may result in enrichment or suppression of a certain protein species. The dynamic range of staining procedures must be considered, since the linear range of most stains and labels does not exceed a factor of 10^3, but protein abundance is far beyond a factor of 10^7. Some of these problems are circumvented by novel approaches. Gerner et al. (2002) developed a method of simultaneous fluorescent staining and radiolabeling of proteins, which allows to compare protein amounts quantitatively and to determine protein half-lives.

Two-dimensional gel electrophoresis offers the possibility of visualizing quantitative results, but the price must be paid that only a limited range of concentrations of the expressed proteins can be stained when compared with direct mass spectrometric approaches (Gygi et al., 2000b; Righetti et al., 2004). Therefore, both approaches will exist most likely side by side for the coming years.

In the future, new methods are envisioned to increase the amount of less abundant proteins in a sample. Such protein amplification machinery should amplify proteins in a way similar to PCR for nucleic acids. If the amplification step can be linearized, a direct quantitation of the protein concentration can be achieved and the protein concentration in the original sample can be calculated.

The enormous dynamic range of protein concentrations that normally exists within the cells makes it obvious that staining methods need to be improved. Such new methods should allow staining proteins in a linear range of at least 10^6, which may come close to the actual protein concentrations. This may help to standardize experimental conditions, as prefractionation and enrichment of some protein species would become obsolete.

4.1.5 Bioinformatics

With the technical improvement of the "hardware" such as mass spectrometers, the gigantic amount of data produced by high-throughput screening methods has to be organized and evaluated. Future developments will not only be based on faster computers with larger memory and storage options, but also on new software tools to interpret proteomic data. One of the most important aims will be to calculate and model tertiary protein structures on the basis of the primary amino acid sequence. This might improve the functional classification of proteins and the search for interacting partners.

5 Conclusion

Proteomics is a screening technology for high-throughput protein analysis. In principle, it permits the description of whole sets of proteins and their functional interaction. Proteomic technology applied to the brain is mainly based on 2D gel electrophoresis for protein separation in combination with MS for protein identification. Although the technological basis of this approach is constantly improving, the application of proteomics for diagnosis and therapy is only starting and remains in the experimental stage. With regard to brain function and biochemistry, proteomics is a useful technology for monitoring the expression and function of a large number of proteins simultaneously, which will further stimulate investigations of the brain and its diseases by the use of proteomics.

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