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Review

Functional and structural proteomics: a critical appraisal

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Abstract

The notion of functional proteomics (description of changes in protein expression during differentiation of cells) and structural proteomics (elucidation of the primary structure of the components in the proteomic pattern) is reviewed. Quantitative and qualitative aspects of the proteome analysis are discussed and evaluated. A list of projects is given that are of high priority for the elucidation of the proteome. This includes, e.g., the study of the repertoire of the proteome and the description of molecular pathways during the clonal cell replication. The paper re-evaluates a number of methodological issues, such as the comparison of the use of Ampholines and Immobilines, various staining procedures and labeling procedures.

Keywords: Reviews; Functional proteomics; Structural proteomics; Two-dimensional gel electrophoresis; Proteins; Ampholines; Immobilines

Contents

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1. Introduction

Since the time when the term proteomics was invented [1–4], the field it covers keeps growing. Although originally ''proteomics'' was meant to be used synonymously to ''2D gel electrophoretic separation and identification of the components displayed in the 2D-representation'', today almost everything in protein chemistry is sheltered under the umbrella of proteomics.

In this review we suggest considering the study of function and structure as two independent tasks and to define the goals of functional proteomics and structural proteomics. The distinction of the two endeavors is not only of a formal nature, but it could help to define areas of research that are unduly neglected. Several such open questions are mentioned in this article.

2. Synopsis of the tools used in functional proteomics

This communication addresses itself to the above-mentioned two categories—with a predilection towards functional proteomics—and methods relevant to the discussed approaches are listed, briefly described and commented upon. Methodological appraisal is given from the viewpoint of the current state of art in our laboratory.

It is now accepted that the technique of two-dimensional gel electrophoresis was developed independently in the laboratories of O'Farrell [5] and of Klose [6]. The first dimension of separation is based on differences in the charge of polypeptides, the second dimension is a molecular separation according to the mass of polypeptide molecules.

2.1. Two-dimensional gel electrophoresis: step one—charge separation

Isoelectric focusing (IEF) is an electrophoretic method that separates proteins according to their charge. Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on their amino acid composition and the pH of their surroundings.

For many years the charge separation matrix was based exclusively on the use of carrier ampholytes (small, soluble, amphoteric molecules) with a high buffering capacity near their p*I* [7], and usually it was a broad range of ampholytes covering p*I* values between 3 and 9 that was used. The era of ampholytes (the LKB brand was named Ampholines) was followed by the development of immobilized pH gradients. Several seminal papers by Righetti et al. [8], Görg et al. [9] and Sinha and Righetti [10] appeared describing this new use. There are several definite advantages of the use of Immobilines—three of them mentioned here. First, due to commercial availability of the Immobiline strips (Pharmacia LKB/Amersham immobilized DryStrip 18-1038-63) a rigorous comparison of results among various laboratories became possible. Second, the loading capacity of the Immobiline strips is considerably higher than that of Ampholine gels. Solubilized cell extract from ten million cells can be conveniently separated by the Immobiline system, while an extract from one million cells would overload the Ampholine gel. Third, narrow range separations became

a reasonably reproducible procedure (the entire length of the Immobiline strip covering only one unit of p*I*). A disadvantage of Immobilines is in the relatively high cost of the Immobilines strips, the limited shelf life of the strips and also various technical inconveniences (a definite view point of my technician), such as the necessity for soaking the strips prior to use or a relatively long separation time (almost double the separation time required by the Ampholine system).

2.2. Two-dimensional gel electrophoresis: step two—size separation

The size separation is performed in a polyacrylamide matrix in SDS milieu. The most common procedure utilizes an acrylamide gradient of 10–20%. Some laboratories use non-gradient slab gels, based usually on 15% acrylamide. Although several attempts were made to commercialize pre-cast gels, financial considerations (and a relatively short shelf life of such gels) drive most of the researchers to cast their own gels.

2.3. Large scale isodalt separation system

Anderson and Anderson have upgraded the method into a robust system capable of performing simultaneous analysis of 20 samples [11–14]. The Ampholine IEF gradient, as well as the gels for the second dimension, are established (cast) in a single step for all 20 samples. For charge separation the tubes are immersed in a large container (2 l) of acidic buffer; for size separation a tank with 30 l cooled electrolyte contents is used. The emphasis of the ISODALT system is not only to achieve reproducibility within the studied set of 20 samples, but also to obtain stable and robust experimental comparisons for consecutive experiments [15,16].

2.4. Cells, clones, cell lines, tissues, body fluids, conditioned media

There are many different protocols for growing cells and clones, and for using solubilized cell extracts for 2D gel separations. One ought to consider two separate environments of applications. In the first, metabolically labeled cells are used, in the second unlabelled cells are employed. In the former, the readouts are autoradiographic or radiofluorographic films (images), in the latter, images of stained gels are obtained [17–19].

When short labeling (in most instances [^{35}S]methionine) is needed, one uses culture medium with depleted cold amino acids (e.g., methionine-free medium), while overnight culture is usually performed in full medium. Very sensitive cultures are labeled for shorter times, while robust cultures can be labeled overnight. Results—in terms of the relative abundance of 2D gel spots—might differ considerably depending on the labeling protocol [20].

Organ cultures (thymic tissue, muscles) are usually labeled overnight [21,22]. Conditioned medium or other secreted material first has to be dialyzed and concentrated, or precipitated and dissolved [22].

Body fluids (serum, tissue exudates) can be obtained from the experimental animal (serum samples and biopsy material from human species) or can be collected upon labeling the entire animal; in vivo labeling is done only for small animals, e.g., mice or frogs [23,24].

2.5. Metabolic labeling and sensitivity of detection

The most common labeling procedures involve incorporation of [^{35}S]methionine into the de novo synthesized polypeptides. In some instances for labeling we use seleno-methionine, ^{3}H-amino acids or ^{14}C-amino acids. Details are given elsewhere [20]. The sensitivity of detection is based on two considerations. First, how much label must be present in a spot in order to be detected as a spot, and second, how close can two spots be localized in order to detect two independent spots?

A spot containing as little as 0.3 dpm can be detected when a radiofluorographic readout is used, while about 3 dpm are required for autoradiographic detection. In terms of amino acid residues, about 100 000 [^{35}S]methionine molecules have to be present in the polypeptide species accumulated in the spots detected by radiofluorography (about one million [^{35}S]methionine residues by autoradiography). Further considerations, especially in terms of the efficiency of labeling) are given in Section 4.5.

The spatial distance between two spots must be about 1 mm in order to be discerned by the naked

eye, or also by image analysis systems, as distinct entities. Autoradiographic readout yields spots with sharper boundaries, and closely localized spots of very different abundances can be well distinguished as distinct spots.

2.6. Sample preparation

It has been recognized that one of the prerequisites for successful analysis is an adequate preparation of samples that are to be applied on the separation matrix. A standard solubilizing buffer, containing NP-40 and urea [13–15] is used in most instances, though a number of recipes were worked out for the solubilization of various ''difficult'' tissues. Several papers from Rabilloud's laboratory describe recipes for many special uses [25–27]. In our laboratory we consider Rabilloud as a kind of ''reference laboratory'' towards good solubilization protocols.

It is our belief that the art of sample preparation continues to be the bottleneck of the whole separation system. Especially problematic is preparing and concentrating samples of secreted proteins (conditioned media), dialyzed and freeze-dried samples, and samples in which there is a predominant accompanying protein, e.g., albumin [22,28,29].

2.7. Staining of gels

Coomassie Blue staining has been developed some 60 years ago by Fazekas et al. [30], and it is used either in its original recipe or as a staining protocol using colloidal Coomassie staining [31]. A clear advantage of colloidal staining is the circumstance that excessive destaining is not necessary. As one can easily guess, colloidal Coomassie staining is considerably more expensive than straight Coomassie Blue application. It should be noted that, as a rule of thumb, only those spots, which are stainable by Coomassie, contain enough protein to enable successful mass spectrometry analysis. The above statement, which is challenged by many laboratories, is dealt with in Section 5.1.

Silver-staining is considerably more sensitive than Coomassie Blue staining. In our hands, very good silver-stained gels are obtained when about one-tenth of the amount of the polypeptide sample that is required for Coomassie staining [32,33] is applied to the separation system.

2.8. Wet gels, dried gels

For drying, we use an apparatus constructed in the workshop of the Basel Institute for Immunology. The apparatus enables simultaneous drying of all 20 gels from the ISODALT system; it is constructed as a system of 10 drawers, each platform accommodates two gels, and during the (overnight) drying cycle it regulates heat and vacuum. The vacuum pump is localized for convenience outside of the lab facilities. Blueprints of the system are available from the author upon request [16]. Wet gels are maintained in a sealed plastic folder, usually for further handling for mass spectrometry.

2.9. Autoradiography, radiofluorography

Metabolic labeling, followed by autoradiography or radiofluorography is the most common readout in functional proteomics, while unlabeled material is used for structural proteomics. Usually several exposures are performed from each gel, and the most suitable ones are selected for the image analysis.

Radiofluorography is based on the impregnation of gels with diphenyloxazole (PPO) and on exposure of films at a temperature of $-70\,^{\circ}C$. The sensitivity of detection is considerably higher than with standard autoradiography, and the exposure time can be shortened by about 8-fold. The O.D. saturation curve for radiofluorography is different from autoradiography, and the two detection systems cannot be combined to evaluate gels within one experiment. As has been pointed out in Section 2.5, autoradiography yields spots with a sharper boundary, and spots localized very close to each other can be better distinguished by autoradiographic than by radiofluorographic methods. For detection of minute spots, radiofluorography is the method of choice.

2.10. Image analysis

There are many sophisticated software systems for the evaluation of 2D gel images. The list in this paragraph is far from complete: Melanie (Geneva Bioinformatics and BioRad Laboratories), Image-

Master (Amersham–Pharmacia Biotech), Phoretix 2D (Phoretix International), Gellab (Scanalytics), Kepler (Large Scale Proteomics), Z3 (Compugen), GD Impressionist (GeneData).

Our laboratory is experienced only in one image analysis system—originally developed by Taylor et al. in Norman Anderson's team at the Argonne National Laboratory, under the name ''Tycho'' [34]. We have used this system from the early stage of its existence, and continue to use the follow-up software ''Kepler'' [35]. We currently use Kepler version 8.0 on a Windows-NT workstation. Each image file contains about 3 MB of data. The image files are processed for noise and streak removal and background correction, and then converted into spot files by spot modeling and fitting. The final ''spot lists'' in which each spot is defined by five parameters: the *x* and *y* coordinates and the spot volume parameters s*x*, s*y*, and amplitude, are compared to each other. At the end of the matching process the *master* pattern contains all the spots occurring in each of the images. All this information on each and all spots is stored in a relational database. It keeps track on all images, spot lists and spot identities, and maintains congruence in the whole system. The Kepler system was meant originally to be commercially available, now it is used only by the LSB organization and for various ongoing collaborative projects by Anderson's group.

The actual laboratory protocols are maintained in a database ''gelscript'' developed by Rovensky and Lefkovits [36].

2.11. Other methods

Methods towards establishing randomly assorted or ordered cDNA libraries, cell free expression systems, restriction endonuclease analysis and techniques for clonal analysis were recently reviewed by Kettman et al. [37], and are beyond the scope of this article.

3. Dichotomy of functional and structural studies

Some 25 years ago, Jerne [38] quoted a saying attributed to Crick [39]: ''If you cannot study function, study structure'', and he noted that those research workers who study function rarely talk to those studying function. In the meantime it became obvious that technological innovations enable a rapid advance in one path of research and then on other occasions in the other path. At present one can observe an enormous boost of innovative approaches, nevertheless the emphasis is towards uses in structural proteomics. The need to put a special effort (and some innovations) into functional proteomics will be felt soon. The identification of protein structure and of gene coding for a given protein might be both the starting and the end point of structural proteomics research, nevertheless the co-regulation, co-expression and mutual relationship(s) of the involved molecules mark the path of functional proteomics.

4. Functional proteomics—applications

Description of changes in protein expression during differentiation, proliferation and signaling of cells, both in qualitative and quantitative terms, falls under functional proteomics. This also includes studies of coordinate expression of genes, as well as elucidation of the sequence of regulatory events during all stages that the cell or an organism undergoes during its entire life span.

4.1. Qualitative and quantitative aspects

If one asks which protein, or which set of proteins, is responsible for a certain biological effect, we are addressing a functional study, and our *hope* is to find an answer, in terms of defining a transcription/translation event responsible for the measured outcome. We know by now that such expectations are in most instances not substantiated. More often it is the case that activation or repression of a gene results in several changes of quantitative nature, extended to several seemingly unrelated components of the studied cell. To understand the basis of the changes, the description of all changes—both in qualitative and quantitative terms—is required.

If a research report states that the expression of a given molecule has been ''shut down'', or ''switched off'', it is almost certain that this—in a strict sense—

is not the case. It is much more probable that 10-fold or maybe 100-fold lesser amounts are expressed, but a certain portion of molecules in the cell still persists. Even in a ''knock-out'' situation, where the gene product should not be present at all, leakiness is often reported—meaning that a certain number of the studied molecules is there. Many events for which we would like to claim a qualitatively ''all or none'' definition, are in fact based on quantitative changes.

Two-dimensional gel electrophoresis is a unique tool that enables the experimenter to obtain a complete view of the changes in the cell. Each spot on the stained gel, or on the radiographic image, provides the necessary characterization of the gene product. The spot is defined by its charge/size separation coordinates, which in the database of the gel is marked by a certain *master number*. Such identification provides a satisfactory definition of the event, irrespective of the availability of structural data. It is the task of the structural proteomics to clarify the structure. To reiterate, it is of utmost importance to elucidate the structure of each of the proteomic components, but the proteomic pattern (especially the quantitative data on the relative abundance of the individual molecular species) provides meaningful information on its own.

4.2. Principal component analysis, cluster analysis and numerical taxonomy

If we accept the notion that discovery of a single regulatory change—even if it represents the main component—does not stand for the entire process, we have to accept that other interpretation methods, aiming at analyzing ''sets of changes'' have to be applied. Principal component analysis, cluster analysis, numerical taxonomy are the terms, one has to get acquainted with [40,41], even if they are not intuitively descriptive. Obviously, in a short conference presentation it is easier to describe a single molecular change, rather than reporting on changes in principal components in hyperdimensional spaces.

4.3. Complete description of the cell

It cannot be the aim of proteomics to describe *every* small detail and *every* small aberration of *every* cell, but we will need to obtain a full description of at least some cell models.

We know the size of the transcriptome, which is about 5000 different mRNA molecules in a model cell (40 000 mRNA in total); in this communication the model of the cell is a lymphocyte in its blast form [42,43]. We know with some precision the number of copies of some messages (and the ratio of the most abundant to the rarest molecules [44]), and we know the total number of polypeptide molecules in the cell is about 10^9 [42–44]. But we do not know which protein is coordinately expressed with which one, and we do not know which proteins are needed at which stage, in what number, or in which compartment. We do not know the number of post-translationally modified polypeptides arising from a given transcript, and we do not know the half-life of these modified entities. In the world of the eukaryotic cells we are at the same stage at which the knowledge on bacteria was before Monod [45] discovered the lac operon. The era of eukaryotic operons has still to come.

4.4. Resting cells and blast cells

There are many other points where we have very little knowledge. We do not know which proteins are needed to ''wake up'' a resting cell (lymphocyte) and which proteins have to be made in order to initiate a transition from a small resting cell into a blast cell. We do not know which events decide upon the fate and destiny of the cell in terms of its ''being associated to self-renewing cell pool'' before turning into a different cell and finally into a terminal cell. We know very little about traffic of molecules within the cell, and we know nothing about the fate of the proteins upon cell death (neither apoptotic nor necrotic).

One could make a long list of facts that need to be known for a true understanding of biological systems under physiological or pathological conditions. We do not know whether during the clonal proliferation each round of cell division uses the same genetic program as the previous one. We do not know whether there is a ticketing mechanism for admittance to the next generation. We do not know whether the so-called Hayflick phenomena [46] are molecularly definable or not. Questions and more questions

can be asked, and meaningful answers start to appear.

4.5. Repertoire of the proteome

As much as the immunological question was not solved before we knew how large the antibody repertoire of an immune competent animal was, cell biology will remain incomplete before clarifying the repertoire of the proteome. How many and which proteins are produced within one species, one individual, a tissue, a cell? We refer here to Norman Anderson's Human Protein Index, as a first approximation to the problem [47].

The scientific community is certainly not discouraged by the finding that ''only'' some 45 000 genes are present in the human genome [48,49]. Due to a profoundly large number of possible post-transcriptional and post-translational changes we shall find that the 45 000 genes are only the basic blueprint for an excessive amplification of final products. The following estimate (for the human species) might serve for testing the true counts, hopefully achieved in a not too distant future (the estimates do not include immunoglobulin molecules, though they do include all such changes that alter the tertiary structure of the molecules):

(a) proteome of a species, 10^8 polypeptides;
(b) proteome of an individual during the entire life span, 10^7 polypeptides;
(c) proteome of an individual as a snap shot, 10^6 polypeptides;
(d) proteome of a cell (model blast cell) 5×10^3 polypeptides.

4.6. Some time ago, before the term proteomics was invented

In 1990 we coined, for a project briefly described below, the term ''proteinpaedia'' [50,51]. Proteinpaedia was supposed to be a list as well as a collection of cDNA molecules and their transcriptomic and translatory counterparts (2D gel coordinates included), with the description of how the entities are related to each other. Proteinpaedia was based on a recombinant phage expression library, where a complete 2D gel pattern of lymphocyte gene products has been recorded. The library was based on randomly assorted recombinant phages, distributed in so-called sectors, each sector having a defined set of cDNA clones [51,52].

Conceptually, proteinpaedia was derived from a Poissonian concept previously applied for the limiting dilution analysis of cells in the immune system [53,54], where randomly assorted lymphocyte clones are distributed in culture wells in the same manner as cDNA clones are distributed in the ''sector collections''. The presence/absence of the assorted entities follows the same Poissonian rule.

Later the use of definite collections of cDNAs was extended towards developing ordered libraries, organized into three-dimensional pools, in which position of any clone is defined by the pooling coordinates [55].

5. Structural proteomics

The aim of structural proteomics is to identify the molecular structure, i.e., the amino acid sequence of the protein entities involved in a given process and to relate this information to the database of identified genes. The most powerful method, one that revolutionized proteomics is mass spectrometric analysis [56].

5.1. Mass spectrometry—spot by spot

Mass spectrometry made it possible to establish molecular structures of a large number of 2D gel spots. Although the methodology is a highly sophisticated one, and all in all it is an expensive undertaking, this approach has no competitive alternative. Admittedly, it is a ''one by one'' analysis, but due to the high throughput system based on robotic support, it is capable of producing and integrating an enormous amount of data in a reasonably short time.

There is an ongoing discussion whether the methodology is suitable in finding also rare components, such as which are present at an abundance of only 10 polypeptide copies per cell. Gradually a consensus starts to take shape: standard proteomic procedures based on 2D gels of entire cell extracts provide results for the most abundant set of proteins, while

material from subcellular fractions furnishes data for low abundance proteins. Special enrichment procedures will be needed for the above-mentioned example of the abundance of 10 polypeptide copies per cell. If indeed 10^{11} polypeptide copies extracted from a spot yield usable MS spectra, an extract of 10^{10} cells might be needed to enter the fractionation procedure.

5.2. Mass spetcrometry—entire 2D matrix

Another way to go about this, is an extension of the MS methodology to analyze the entire matrix of the 2D gel. This approach, currently under scrutiny in Hochstrassers' laboratory [56] will probably give the final breakthrough, since in this approach the MS spectra are inspected in the context of all neighboring MS spectra. This procedure, considered conceptually, will enable to read results also from overlapping spot entities.

It is probable that—due to the enormous computing power of modern computers—it will be possible very soon to deduce polypeptide structures from MS spectra obtained from polypeptide mixtures. Each month thousands of spectral prototypes are added to the databases of each MS laboratory, such that each new sample has a higher chance of obtaining an instantaneous structural identification.

6. Jerneian questions

Some 30 years ago, Jerne, in a paper ''Complete solution of immunology'' [57] said that ''there are things, which we do not want to know''. We do want to know a lot about the geography of Australia, but we do not need to know where each pebble on each coast lies (quoted from memory). Jerne was a scientist, who knew what question could and should be asked. Indeed, we do not need to know everything about everything, but I believe that at least for some model cases we need to know:

(a) How many and which genes—and in which order—are activated in a cell?

(b) How many polypeptide molecular species, and in what amounts are synthesized?

(c) What kind of post-translational modifications occur on the nascent proteins?

(d) What is the half-life of the protein molecules and how does this affect the initiation and termination of any given function?

(e) What are the rules for moving some proteins into and out of any cellular compartment?

We might agree that at least some of the above questions are of importance.

7. Concluding remarks

The suggestion to distinguish between functional and structural proteomics might seem to be of minor importance in the context of the ongoing proteomics projects. It is the opinion of this author and his laboratory, that this distinction will be helpful, since those who study structure might become aware that functional proteomics could provide means to distinguish—poetically speaking—pearls from pebbles. Those who study function will be less irritated by deconstructing the global 2D gel picture into so many mosaic stones. Continuing the parable, the mosaic stones are often indistinguishable pearls and pebbles, and it will be that what is a pebble for one scientist will be a pearl for the other scientist.

And the so-called ''non-hypothesis driven'' research will turn out to be the refrain of Pablo Picasso ''Je ne pas cherche, je truve'' [58].

8. Further reading

This review article meant to illuminate the field of proteomics from a somewhat different angle than is the case for traditional approach. The arguments would do injustice to all those efforts not referred here. Therefore I wish to draw attention to several classics in the field. I shall start with the two books by Righetti [59,60] which are a must for both the beginner and the advance reader. The books give historical overviews, solid basis for understanding the theory and the practice, and are intelligent reading in their own right.

Then there are the books from the new era, when the magic word ''proteomics'' started to be used by everyone—not only by scientists but also by opinion leaders in finance and politics. Among the books which should not be missed on the shelf of a

‘‘proteomician’’ are those written or edited by Wilkins et al. [61], Rabilloud [62] and Righetti et al. [63], while the mass spectrometry approach and other procedures of protein characterization are given in an excellent manner by James et al. [64] and Kellner et al. [65].

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