Preface

Post-translational Modifications of Proteins: Tools for Functional Proteomics, Second Edition, is a compilation of detailed protocols needed to detect and analyze the most important co- and post-translational modifications of proteins. For reasons of simplicity, although not explicitly mentioned in the title, both kinds of modifications are covered, regardless of whether they occur during or after biosynthesis of the protein. My intention was to cover the most significant protein modifications, focusing on the fields of protein function, proteome research, and characterization of pharmaceutical proteins.

The majority of all proteins undergo co- and/or post-translational modifications. The protein's polypeptide chain may be altered by proteolytic cleavage, formation of disulfide bonds, or covalent attachment of phosphate, sulfate, alkyl groups, lipids, carbohydrates, polypeptides, and others. Knowledge of these modifications is extremely important, because they may alter physical and chemical properties, folding, conformation distribution, stability, activity, and, consequently, the function of the proteins. Moreover, the modification itself can act as an added functional group. Examples of the biological effects of protein modifications include: phosphorylation for signal transduction, ubiquitination for proteolysis, attachment of fatty acids for membrane anchoring or association, glycosylation for protein half-life, targeting, cell–cell and cell–matrix interaction, and carboxylation in protein–ligand binding, to name just a few. Full understanding of a specific protein structure–function relationship requires detailed information not only about its amino acid sequence, which is determined by the corresponding DNA sequence, but also on the presence and structure of protein modifications. Consequently, analysis of post-translational modifications of proteins is essential for proteomic research, the development of new drugs, and for the production, registration, and monitoring of therapeutic pharmaceutical proteins.

In general, post-translational modifications of proteins can be classified according to their chemistry or the targeted amino acid. They can be subdivided into reversible or irreversible reactions, enzymatic or nonenzymatic reactions, according to their subcellular location or functional aspects of the modification. Though the organization of the chapters considers both the frequency and the chemical nature of the particular post-translational modification, it still remains arbitrary. The individual chapters of this book provide detailed step-by-step instructions for

the analysis of the most important protein modifications, e.g., the assignment of disulfide bonds in proteins (Chapter 1). The detection and analysis of protein phosphorylation by selective fluorescent staining in 2D-gels and by advanced mass spectrometry, respectively, is covered by Chapters 2 and 3. Chapters 4 to 7 describe analysis of protein sulfation, α-amidation, γ-glutamate, ß-hydroxyaspartate, and lysine hydroxylation. Protein ubiquitination, sumoylation, and ISGylation are covered by Chapters 8 to 10, analysis of protein methylation and acetylation by Chapter 11. Methods for analysis of lipid modifications to both the carbohydrate and lipid portion as well are given in Chapters 12 and 13 on S-acylation and glycosylphosphatidylinositols respectively. Chapters 14 to 21 describe analysis of protein glycosylation in great detail. Starting with the detection of protein glycosylation (Chapter 14), analysis of carbohydrate composition (Chapter 15) cleavage, labeling, separation, and sequence analysis of *N*-linked glycans are described (Chapters 16 to 18). Analysis of protein *O*-glycosylation in general and specific detection of *O*-linked N-acetyglucosamine residues follow (Chapters 20 and 19, respectively). Analysis of *O*-glycosidically linked *N*-acetylglucosamine (O-GlcNAc) deserves special mention. *O*-GlcNAc is a transient modification, which is involved in several cellular functions as transcription, translation, nuclear transport, and cell signalling. Because of its exceptional position within the glycosylation of proteins it is treated in a separate chapter. Chapter 21 provides a method to analyze the oligosaccharides that are present at specific single glycosylation sites in a protein. Chapters 22 to 24 give practical approaches, i.e., how to analyze and monitor glycosylation of recombinant proteins from different cell lines. Finally, a topic of general interest is treated in the last chapter. Chapter 25 describes the use web-based protein databases for analysis of post-translational modifications of proteins. Web-based databases give information on protein modifications and allow the prediction of post-translational modifications on yet uncharacterized proteins, based on the fact that post-translational modifications occur at specific amino acids, amino acid sequences, or specific 3D-structures of the protein, respectively.

Let me give special mention to two areas of research of high current interest: the fields of (1) proteomics and (2) the characterization of biological pharmaceuticals. (1) With respect to proteomics, research in the field of genomics has lead to knowledge of the complete human DNA sequence. Measurement of the mRNA pool at a specific status of the cell, the "transcriptome," was found to not necessarily reflect the cells' actual protein expression pattern. In proteomic research, the description of expression levels of proteins related to a defined cell or tissue status will be incomplete without knowledge of post-translational modifications of those proteins. The increasing interest in post-translational modifications of proteins in this field is reflected by use of the term "phosphoproteomics." Phosphoproteomics describes the analysis of the sites and amount of protein phosphorylation under different biological conditions. (2) An additional important practical application of post-translational modification analysis is to ensure product quality of therapeutic pharmaceutical proteins. The exact structure of a protein pharmaceutical cannot be defined without knowledge of all post-translational modifications. Recombinant proteins intended for therapeutic use in humans must be accorded particularly

thorough investigation. Product quality depends on accurate post-translational modification in the respective expression system during production, e.g., insect, several mammal, or human cell lines. Note that different expression systems may vary in their ability to carry out post-translational modifications and that the applied cell-culture conditions also influence these modifications. Thus, post-translational modifications of recombinant proteins have to be monitored during production and documented for registration. In their guidance Q6A for the pharmaceutical industries, the international conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) states, that "For desired product and product-related substances, details should be provided on primary, secondary and higher-order structure; post-translational forms (e.g., glycoforms); biological activity, purity, and immunochemical properties, when relevant." Consequently, almost each and every post-translational modification of a protein is of concern for the regulatory agencies. Moreover, glycoengineering, the directed modification of protein glycosylation, or the artificial attachment of polymers to therapeutic proteins demand analytical tools for their characterization as well.

Growing knowledge of the biological roles of protein modifications, on the one hand, and the development and availability of sophisticated, sensitive analytical methods on the other hand, are already leading to increased interest in co- and post-translational modifications of proteins. *Post-translational Modifications of Proteins: Tools for Functional Proteomics* intends to serve as practical guide for researchers working in the field of protein structure–function relationships in general, in the rapidly growing field of proteomics, as well as scientists in the pharmaceutical industries.

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Detection of Post-translational Modifications by Fluorescent Staining of Two-Dimensional Gels

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Summary Post-translational modifications (PTMs) are key to the regulation of functional activities of proteins. Quantitative and qualitative information about PTM stages of proteins is crucial in the discovery of biomarkers of disease. Recent commercial availability of fluorescent dyes specifically staining PTMs of proteins such as phosphorylation and glycosylation enables the specific detection of protein regulations taking place with respect to these modifications. Activity and molecular and signalling interactions of many proteins are determined by their extent of phosphorylation. In our search for biomarkers of neurodegenerative diseases such as Multiple Sclerosis (MS), using its animal model, Experimental autoimmune encephalomyelitis (EAE), we have applied the phopshorylation specific fluorescent dye, ProQ Diamond, to study changes taking place in the phosphoproteome. Subsequent Colloidal Coomassie staining of the same gels detects the changes at the whole proteome level. We have detected many changes taking place in the CNS tissue of the EAE animals at the whole proteome as well as at the phosphoproteome level that has given valuable insights into the pathophysiological mechanism of EAE and possibly also MS.

Key Words Phosphoproteome; ProQ Diamond; 2D gel electrophoresis; fluorescent stain; in-gel digestion; peptide extraction.

1 Introduction

The analysis of post-translational modifications is of high significance in proteomic studies aimed at the discovery of protein markers relevant in the pathogenesis of diseases. Although limited in the coverage of the whole proteome, one of the main strengths of 2-dimensional polyacrylamide gel electrophoresis (2D PAGE) is the ability to visualize protein isoforms. However, because of their very low stoichiometry, the post-translationally modified isoforms often remain undetected on 2D gels using classical staining methods. Commercially available fluorescent stains such as, ProQ Emerald, (Molecular Probes) and ProQ Diamond, (Molecular Probes)

have facilitated the specific detection and identification of glycosylation and phosphorylation of proteins separated on 2D gels, respectively. Proteins separated on 2D gels for proteomic analysis can be stained first by ProQ and subsequently by Colloidal Coomassie stain or any other protein stain.

ProQ Diamond stain binds specifically to proteins with phosphate groups on serine, threonine, and tyrosine residues. Because of the fluorescent nature ProQ Diamond stain can detect phosphorylated proteins present in as low as 4 ng per spot. The staining intensity correlates with the number of phosphate groups present in the respective proteins *(1,2)*. This high sensitivity is especially very critical in the case of phosphorylated proteins because of their very low abundance *(3,4)*. ProQ Diamond staining allows the comparative expression profiling of the phosphoproteome both in a quantitative and qualitative manner. Moreover, its high sensitivity increases the proteome coverage. As the dye binds noncovalently to the phosphate groups, it is compatible with subsequent mass spectrometric analysis.

Mouse CNS tissue protein extracts are separated on 2D gels and stained for phosphoproteome and whole proteome (Figs. 2.1a and 2.1b). The two images are digitally colored and overlaid (Fig. 2.1c) to determine the relative position of the phosphoproteins on the whole protein stained image. The spots of interest are excised and prepared for mass spectrometry analysis. Thus, this method detects in parallel the expression level changes and altered phosphorylation modifications taking place under different physiological conditions (Figs. 2.2 and 2.3).
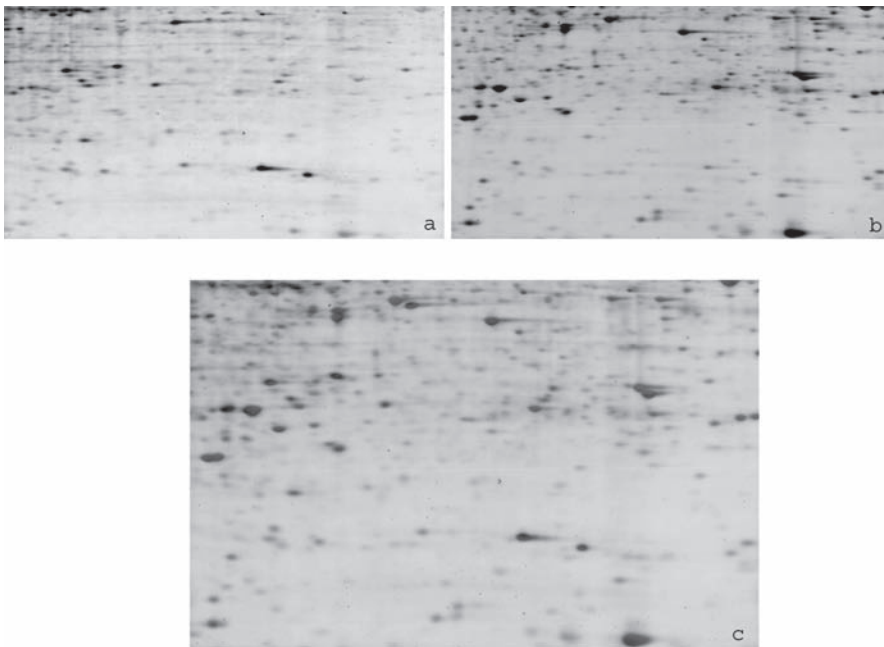
Fig. 2.1 Phosphoproteome and whole proteome. (**a**) ProQ Diamond stained image of mouse spinal cord 2D gel. (**b**) Image of the same gel stained with Coomassie Blue. (**c**) The 2 images overlaid on top of each other. Image C is used to determine the relative position of the ProQ Diamond stained spots on the Coomassie stained image for spot picking
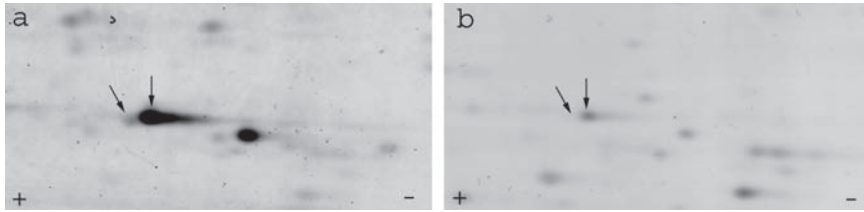
Fig. 2.2 (**a**) ProQ Diamond stained gel image. (**b**). Same gel stained subsequently with Coomassie stain. The arrows indicate a protein that migrates at two different positions on a 2D gel. The more acidic spot is visible only on the ProQ Diamond stained image
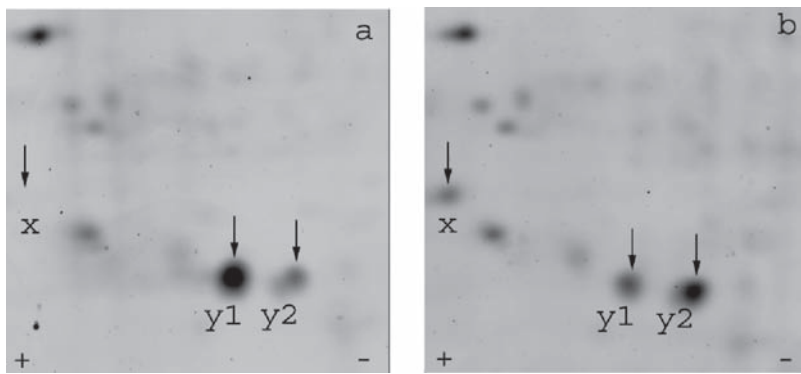
Fig. 2.3 Quantitative and qualitative differences of the phoshoproteome between control and EAE mouse brain. (**a**) 2D gel image of EAE brain proteins stained with ProQ. (**b**) 2D gel image of control brain proteins stained with ProQ. Spot **x** is up-regulated in EAE, an example for a quantitative difference in expression. Spots **y1** and **y2** represent the same protein. It differs between diseased and control animals in its extent of phosphorylation, a typical example of a phosphorylaton change, where the protein moves more to the acidic end

2 Materials

2.1 Sample Preparation of Brain and Spinal Cord Sample for 2-Dimensional Electrophoresis

Unless otherwise mentioned all reagents are purchased from Bio-Rad, Hercules, CA

1. Isoelectric focussing (IEF) buffer: 7M urea, 2M thiourea, 100 mM dithiothreitol (DTT), 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesul-fonate hydrate (CHAPS), 0.05% biolytes 3–10, 0.001% (v/v) bromophenol blue (for color). Prepared and stored as 1-mL aliquots at −80°C. Once thawed should not be frozen again (*see* **Note 1**).
2. Protease inhibitors: All protease inhibitors are added to the IEF buffer in 1 × con-centration just before use.

   a. Pepstatin (Roche): 1000× stock prepared by dissolving 1 tablet in 1 mL ethanol and stored at −20°C up to 3 mo.

   b. Complete (Roche): 25× stock prepared by dissolving 1 tablet in 2 mL Double distilled water and stored in aliquots at −20°C up to 3 mo.

   c. Phenylmethylsulfonyl fluoride (PMSF), (Roche):100 mM stock (100×) prepared in methanol or ethanol and stored at 4°C.

3. Tissue sample grinding kit (GE Healthcare Amersham Biosciences) (*see* **Note 2**).

2.2   2-Dimensional Gel Electrophoresis

2.2.1   Isoelectric Focusing

1. IPG strips of desired pH range (4–7 pH range for brain tissue and 5–8 pH range for spinal cord).
2. Filter wicks.
3. Isoelectric focusing apparatus.
4. Equilibration trays.

2.2.2   Equilibration

1. Equilibration buffer base (EQB): 50 mM Tris-HCl, pH 8.8, 6M urea, 2% sodium dodecyl sulphate (SDS), 20% (v/v) glycerol. Store as 20- and 40-mL aliquots at −20°C. While aliquoting, the solution should be constantly stirred using a magnetic stirrer. Glycerol will otherwise accumulate at the bottom. Thaw before use and vortex to get a clear solution.
2. Equilibration buffer 1: EQB containing 2% (w/v) DTT. Dissolve few hours before use at room temperature and keep at dark. Working volume is at least 6 mL per gel strip.
3. Equilibration buffer 2: EQB containing 2.5% (w/v) iodoacetamide (Bio -Rad). Dissolve few hours before use at room temperature and keep at dark. Working volume is at least 6 mL per gel strip.

2.2.3   SDS PAGE

1. Tris buffers: 1.5M Tris-HCl, pH 8.8, and 0.5M Tris-HCl, pH 6.8. Store at room temperature.
2. 10% SDS. A ready made stock of 20% w/v SDS is diluted 1:2 with water and stored at room temperature.
3. Thirty percent acrylamide/bis solution (37.5:1 with 2.6% C) (Genaxxon) (*see* **Note 3**).
4. *N,N,N,N′*-Tetramethyl-ethylenediamine (TEMED).

5. Ammonium persulfate (APS): Prepare 10% w/v solution in water before use.
6. Water-saturated isobutanol. Shake equal volumes of water and isobutanol in a glass bottle and allow to separate overnight. Use the top layer. Store at room temperature.
7. Agarose overlay buffer: 0.5% (w/v) agarose is dissolved in Tris glycine SDS (TGS) running buffer by boiling in a microwave. Few drops of bromophenol blue are added to the buffer for color. Store at 4°C. Melt in a microwave before use and maintain at 60°C before use.
8. Running buffer (10×): 250 mM Tris, 1.920M glycine, 1% (w/v) SDS pH 8.3. Store at room temperature.
9. Prestained molecular weight markers.
10. Hinged spacer plates.
11. Gel casting chamber.
12. Gel combs for 2D gels with 1 reference well (Protean plus comb, Bio –Rad).

2.3   ProQ Diamond Staining

1. Fixing buffer: 10% acetic acid, 50% methanol. Stored at room temperature or prepared before use (*see* **Note 4**).
2. ProQ Diamond stain (Molecular Probes), store protected from light at 4°C (*see* **Note 5**).
3. Destain 1: 20% Acetonitrile (ACN) and 50 mM sodium acetate pH 4.0 (*see* **Note 6**). For stock buffer solution, dissolve IM sodium acetate in double distilled water and adjust the pH to 4.0 using fuming hydrochloric acid (HCl) and store at room temperature.
4. Staining trays compatible with methanol

2.4   Colloidal Coomassie Staining

1. Colloidal solution: 17 mM ammonium sulphate, 2% phosphoric acid and 34% v/v methanol (*see* **Note 7**).
2. R-250 Brilliant Coomassie, (Sigma).

2.5   Spot Processing

1. Destain 2: 1:1 solution of 20 mM NH$_4$HCO$_3$, pH 8.00, and 100% ACN. Mix equal volumes and leave and store at room temperature.
2. 1 mM NH$_4$HCO$_3$ pH 8.0 and trypsin (Sequencing grade modified trypsin, Promega). Dissolve trypsin in 1 mM NH$_4$HCO$_3$ at 1 µg/µL concentration and store in 5-µL aliquots at −20°C.
3. 2% trifluoroacetic acid (TFA), (Merck) and 5% formic acid (HCOOH), (Merck).