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Utilisation of Chromatographic and Electrophoretic Separation Techniques for the Detection of Protein Post-Translational Modifications

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Abstract

Aberrant post-translational modifications (PTMs) of proteins and the resultant disturbances of cellular signaling pathways typify many disease states. Hence the detection, localisation, and quantitation of PTMs represent important elements of disease analysis. Herein, chromatographic and electrophoretic methodology that may be exploited to first detect the protein(s) that is post-translationally modified, determine the site(s) of the PTM, quantify the stoichiometry of the modification, and then evaluate its stability is discussed. For proteins at which multiple PTMs occur, synthetic peptides that only encompass selected modification site(s) are useful for restricting analysis to specific PTMs. Suggestions for rational peptide design for studying discrete PTMs are detailed. Finally, intrinsic to these analyses of PTMs is the requirement of an evaluation of the functional outcome of the PTM.

Introduction

It is likely that all proteins undergo a level of post-translational modification that may influence protein structure and function. This PTM may arise enzymatically, such as the burgeoning list of amino acid modifications that trigger, for example, changes to enzymatic activity, cellular localization, or provide the signal for targeted proteolytic degradation. Additionally, proteins undergo non-enzymatic PTMs such as isomerisation, deamidation, or glycation, which may also influence protein structure and function.

Some PTMs may be reversible; indeed enzymatic modification as reversible phosphorylation provides a molecular switch that can be used to regulate protein activity. Irreversible PTM constitutes a more permanent change in protein structure and may result in irretrievable loss of protein function.

Protein PTMs may arise from orchestrated cellular events (for example, phosphorylation), or alternatively, from directed or inadvertent exposure to the external environment; for example, environmental chemicals such as pesticides that can be taken up by routes including inhalation, or skin contact, traverse cellular membranes to covalently adduct cellular protein targets.

Methodology

In an attempt to understand the complex array of protein PTMs, there is the primary need to determine which protein is modified. The principal laboratory techniques employed to identify post-translationally modified proteins include the use of radiochemical tracers, affinity-based activity ligands, selective visualization methods, or mass spectrometry. In all of these techniques the method of analysis should ideally be designed to be suitably specific to solely monitor the target(s) of the PTM.

Secondly, to initiate structure-activity analysis, the site(s) of PTMs need to be investigated. The major laboratory methods employed for this include the use of radioactive tracers, mass spectrometry, and Western blotting with PTM-specific antibodies. Additionally, one should consider the stoichiometry of modification, and similar methodology is often employed for this purpose. In addition to the requirement to evaluate the stoichiometry, there is the need to assess the stability of the modification, and finally, one needs to consider the biological outcome of the PTM.

Results and Discussion

As an example of detection and purification of protein targets of selective PTMs, we have examined the cytosolic protein targets of several commercial organophosphorus (OP) pesticides commonly employed within the UK and USA. OP pesticides (when in their active oxon forms) possess a canonical chemical signature of a central phosphorus atom double bonded to oxygen, and bonded to R1 and R2 groups, and a 'leaving' group. The primary target of OP pesticides or nerve agents is acetylcholinesterase (AChE) within nerve synapses. Organophosphorylation of AChE produces a protein adduct that is resistant to hydrolysis; in addition if the adduct undergoes dealkylation (termed 'ageing'), this results in further resistance to hydrolysis. Hence this constitutes an essentially irreversible PTM. AChE is a member of the serine hydrolase family of proteins, which typically possess an active site triad of amino acids that includes a serine residue. This active site serine residue is the amino acid that undergoes OP adduction. Likewise, other proteins that contain a similar consensus sequence for modification, such as serum albumin, are also susceptible to adduction by OPs.

We have exploited the use of a radiolabelled (tritiated) organophosphate, ^{3}H-diisopropylfluorophosphate (DFP), to provide the chemical signature of the OP pesticides. Incubation of ^{3}H-DFP with cytosolic proteins resulted in the labeling of only tissue active serine hydrolases, or proteins that possess similar chemical reactivity. Our strategy for detection of protein targets adducted by pesticides was to preincubate cytosolic proteins with a non-radiolabelled pesticide prior to incubation with the broad serine hydrolase inhibitor 3H-DFP. If the non-labeled pesticide bound to a protein target, there was a reduction in

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subsequent radiolabelling of this protein with 3H-DFP. To characterize these new pesticide targets, proteins were resolved by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then autoradiography was used for protein visualization [1-4].

SDS-PAGE thus provides a convenient method of initial characterization of unknown protein targets via their denatured molecular weight. However, protein radiolabelling and coupling to suitably sensitive autoradiography can be several orders of magnitude more sensitive than protein staining methods such as Coomassie blue or even silver staining. Thus chromatographic purification techniques provide an invaluable means to enrich proteins of interest to a level for protein staining and mass spectrometric identification.

For purification of cytosolic ^{3}H-DFP (and pesticide) protein targets we have utilised MONO Q column chromatography as the first step for protein purification. MONO Q, a positively charged quarternary ammonium compound, is a useful start point for purification of cytosolic proteins since most cytosolic proteins are predicted to be negatively charged at physiological pH [5], and will therefore associate with a positively charged column matrix.

Other methods for detecting the target proteins of PTMs include the use of activity-based affinity ligands which will react with active members of a protein family. For example, fluorophosphonates have been used in the study and identification of novel targets of pesticides [6].

There are also a range of chromatographic and electrophoretic separation techniques which can be used to determine the amino acid site(s) of PTM. For example, after radiolabelling the sites of PTM in a native protein, two-dimensional PAGE can be employed to resolve proteins, and the resultant autoradiogram can be used as a template with which to superimpose the positions of post-translationally proteins to direct their extraction and identification by mass spectrometry [7]. Alternatively, after radiolabelling the sites of PTM in a native protein, enzymatic cleavage can be used to liberate peptides. Resolution of these peptides by, for example, two-dimensional thin layer chromatography (2D-TLC), or reverse phase high performance liquid chromatography (RP-HPLC) provides a means to track the localization of the radiolabel to distinct peptides. Synthetic peptides can similarly be exogenously radiolabelled, and resolved under identical conditions to ascertain if the synthetic peptide exhibits identical chromatographic behavior to peptides from the native protein to enable PTM site identification [8-10]. Peptides may, however, possess more than one potential site of PTM, so radiosequenation of the peptide, site directed mutagenesis, or further peptide fractionation may be needed to complete unequivocal determination of the PTM site [8].

Additionally, the site(s) of PTM may be confirmed using PTM-specific antibodies and Western blotting. Commercial antibodies directed against proteins containing specific PTMs are now commonplace. Also, with the advent of sensitive mass spectrometry instruments, direct detection of the proteins that are modified, and/or the sites of PTMs can be undertaken after suitable sample enrichment (reviewed in [11]).

Many (if not all) proteins undergo PTMs at multiple sites, although some modifications may not greatly influence functional activity of the protein. Native or recombinant proteins have been utilized to provide a molecular target to study the PTM of interest, but if multiple PTMs are present, then a method of discernment is required to restrict the analysis to the PTM of interest. The production of shortened recombinant proteins or synthetic peptides that only encompass selected modification site(s) are useful reagents for restricting analysis to specific PTMs. Our experience in the design of peptide mimics has led us to suggest the following 'rules' for rational peptide design:

1. The synthetic peptide sequence should encompass several amino acids upstream and downstream of the PTM site in order to provide any localized primary consensus sequence that directs the PTM. Hence a minimum peptide length of 6-10 amino acids may be required.

2. To enable subsequent monitoring of peptide behavior and a direct comparison with the full-length native protein, synthesis of a peptide that mirrors an enzymatic cleavage product of the native protein is advised. For example, proteins can be readily cleaved at the carboxyl side of arginine or lysine residues using trypsin, hence a synthetic peptide based upon a trypsinolysis fragment will facilitate mapping to a similarly digested native protein. We have used this approach for radiolabelling whole protein and synthetic peptides *in vitro* [8,10]. If enzymatic (or chemical) cleavage methods are to be used, one should also consider if the synthetic peptide sequence contains a blocked or weak cleavage site, for example, the presence of arginine–proline bonds, or lysine-proline bonds that are resistant to trypsinolysis. Similarly, one should consider the potential for inhibition of enzymatic digestion as a consequence of the PTM; for example acetylation or di-, or tri-methylation of lysine or arginine residues also renders the modified amino acid resistant to trypsin digestion.

3. For assessment of PTM using radiolabelling, one should consider the optimal method of removal of extraneous radiolabel. Separation of incorporated from unincorporated radiolabel will facilitate visualization (and quantification) of protein modification after autoradiography, and an accurate quantitative determination of radiolabelling and stoichiometry of modification by liquid scintillation counting (LSC). Examples of the techniques used for removal of unincorporated radiolabel include SDS-PAGE, RP-HPLC, or ion exchange [1-4,7-10,12].

The charge and length of the peptide will also impact on the method used for its separation from extraneous radiolabel and detection. For example, peptides with net positive charge at the pH used for analysis may be separated from unincorporated radiolabel by binding and association with negatively charged media. Similarly, peptide length will influence the methods available to separate the radiolabelled peptide from unincorporated radiolabel. For example, radiolabelled peptides with a molecular weight of greater than 1,000 Da (typically > 10 amino acids in length) can be resolved from phosphoproteins, and from unincorporated radiolabel ($[\gamma$-^{32}P]-ATP) using three-phase peptide gels [13].

Once the site of PTM is established, there is a need to consider the stoichiometry of modification, *i.e.* how many of the molecules of a population are affected by the PTM. Radiolabeling methods may also provide a convenient means to quantify PTM stoichiometry. For example, the level of radioactivity incorporated into a protein via a PTM can be quantified directly by LSC. From utilization of a radiolabel with known specific activity and a known concentration of the protein of interest, the number of molecules radiolabelled (stoichiometry of modification) can be determined. Alternatively, PTM-specific antibodies and Western blotting can provide a quantitation of the level of PTM of a specific protein and be compared to the Western blotting

signal generated from the total protein of interest. Quantitative PTM determination by mass spectrometry may also be possible.

Additionally, one should consider the stability of the PTM; is the PTM actively (enzymatically) removed? For example, a phosphate moiety may be (catalytically) removed by the action of a phosphatase. Alternatively, is the PTM spontaneously hydrolysed, and thus removed at a steady rate, or is the PTM irreversible, in which case it should persist for the lifetime of the protein, and follow similar protein half-life kinetics. Again, protein radiolabelling may provide quantitative measurements of the PTM stability. As an example of this, we investigated the OP adduction of serum albumin, and were able to show that the protein adduction followed the half-life of the protein, and was therefore an essentially irreversible PTM [2].

Finally, one will need to consider the functional outcome of the PTM. Is the PTM benign and without significant influence on protein structure and function, or does the PTM have a profound effect on the protein's activity, binding with other proteins, cellular localization, etc.?

In summary, PTMs comprise an every expanding list of protein modifications that can have an impact upon protein structure and function. Moreover, changes in PTMs may arise independently from alterations in protein levels, and hence may be missed by conventional differential proteomics. However, by establishing suitable methods for studying PTMs we can begin to dissect their role in patho-physiological processes.

Acknowledgements

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