[27] Identification of Histidine Phosphorylations in Proteins Using Mass Spectrometry and Affinity-Based Techniques

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Abstract

Histidine phosphorylation plays a key role in prokaryotic signaling and accounts for approximately 6% of the protein phosphorylation events in eukaryotics. Phosphohistidines generally act as intermediates in the transfer of phosphate groups from donor to acceptor molecules. Examples include the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) and the histidine kinases found in two-component signal transduction pathways. The latter are utilized by bacteria and plants to sense and adapt to changing environmental conditions. Despite the importance of histidine phosphorylation in two-component signaling systems, relatively few proteins have so far been identified as containing phosphorylated histidine residues. This is largely due to the instability of phosphohistidines, which, unlike the phosphoesters formed by serine, threonine, and tyrosine, are labile and susceptible to acid hydrolysis. Nevertheless, it is possible to preserve and identify phosphorylated histidine residues in target proteins using appropriate sample preparation, affinity purification, and mass spectrometric techniques. This chapter provides a brief overview of such techniques, describes their use in confirming histidine phosphorylation of a known PTS protein (HPr), and suggests how this approach might be adapted for large-scale identification of histidine-phosphorylated proteins in two-component systems.

Introduction

Reversible phosphorylation is one of the most common and most important mechanisms by which the structure and function of a protein can become post-translationally modified. It is estimated that up to 30% of all proteins may be phosphorylated at any given time (Cohen, 2000). Site-specific phosphorylation of proteins affects localization, turnover, and enzymatic activity, as well as interactions with other proteins and DNA (Patel and Gelfand, 1996; Wolschin *et al.*, 2005). Phosphorylation occurs on the side chains of certain amino acid residues. The resulting phosphoamino acids fall into three categories: O-phosphates, which are formed by serine (Ser), threonine (Thr), and tyrosine (Tyr) and contain phosphoester linkages; N-phosphates formed by

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histidine (His), lysine (Lys), and arginine (Arg), which contain phosphoamidate bonds; and the acyl-phosphate formed by aspartic acid (Asp). The addition and removal of phosphate groups are usually mediated by specific classes of enzymes, such as histidine kinases (phosphorylation of His) and serine/threonine phosphatases (dephosphorylation of Ser and Thr). Phosphohistidines, which are the least stable of the phosphoamino acids, may or may not require histidine phosphatases, depending on the protein and, in particular, the residues adjacent to phosphohistidine (Klumpp and Krieglstein, 2002).

In terms of physiological function, there are two main classes of protein phosphorylation. The first of these encompasses phosphorylation for the purpose of regulating enzymatic activity, and usually involves modification of Ser, Thr, and Tyr residues. The resulting phosphoesters are stable entities, and generally serve to regulate enzymatic catalysis without direct involvement in the catalytic mechanism. Reversible, multisite phosphorylation of Ser, Thr, and Tyr mediates numerous signal transduction pathways in eukaryotic cells (Cohen, 2000). The second class encompasses phosphorylation for the purpose of phosphate group transfer, and is generally restricted to phosphorylation of His residues. Phosphohistidines act as high-energy intermediates in the transfer of phosphate from phosphodonor to phosphoacceptor molecules (Stock *et al.*, 1989), a role for which these labile modifications are well suited. Examples include the bacterial phosphoenolpyruvate:sugar phosphotransferase system (Meadow *et al.*, 1990) and the histidine kinase enzymes found in two-component signal transduction pathways (Parkinson and Kofoid, 1992). The latter are utilized by bacteria, plants, and lower eukaryotes to sense and adapt to changing environmental conditions (Klumpp and Krieglstein, 2002). In bacteria, such adaptations may include changes in motility, cell morphology, and gene expression, as well as the establishment of virulence and antibiotic resistance.

Due to the importance of protein phosphorylation in regulating key biological processes, considerable effort has been put into developing procedures for identifying and mapping sites of phosphorylation, both for individual proteins and on a proteome-wide scale (Beausoleil *et al*., 2004; de la Fuente van Bentem *et al*., 2006; Nühse *et al*., 2003). However, the substoichiometric nature of this modification continues to present major challenges; indeed, the phosphorylated form of a particular protein may represent only a small fraction of its total abundance. Furthermore, many proteins can undergo phosphorylation at different sites (Cohen, 2000), resulting in a number of potential phosphorylated isoforms of which several may be present at any given time. As a result, the full complement of phosphorylated proteins in a cell, tissue, or organism (the phosphoproteome) can be extremely complex.