ANALYSING NATURE WITHOUT COSTING THE EARTH

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MALDI MASS ANALYSIS OF HIGHER-ORDER PROTEIN STRUCTURE

KOMPACT

APPLICATION NOTE

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INTRODUCTION

In their native state, proteins are typically folded into well-defined three-dimensional structures held together by relatively weak intra-molecular forces. Protein function relies heavily on this well-defined structure, and therefore, information on higher-order structure (i.e. tertiary and quaternary) is of fundamental biological importance. MALDI MS in combination with proteolysis, more commonly known as peptide mass mapping, is a method routinely used to probe primary protein structure (amino acid sequence). Here it is shown to be a valuable method with which to investigate higher-order protein structure (tertiary-protein conformation and quaternary-protein-protein interactions).

Peptide mass mapping is routinely being used to examine amino acid sequence for protein identification. In this case it is advantageous to generate peptide fragments which cover the entire sequence of the protein so as to increase the probability of matching the protein in a database search. As a result, proteins are typically denatured prior to digestion. In the present case, however, conservation of the native structure is essential to the analysis, as available cleavage sites are indicative of higher-order structure. For example, a protein in a well folded, globular state will have fewer cleavage sites available than in an unfolded, more linear conformation (Figure 1). Digestions would therefore produce two different sets of peptide fragments and thus different mass maps.

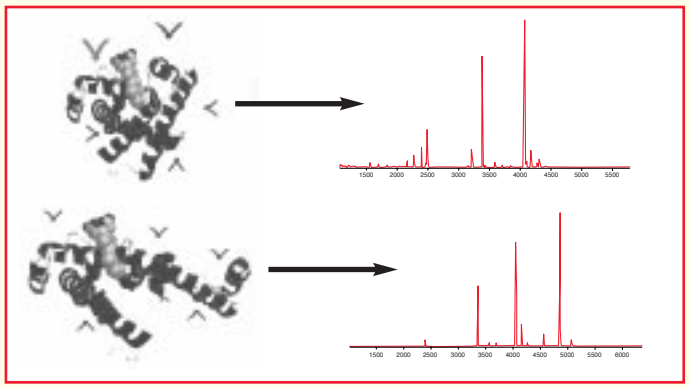

Figure 1. Strategy for probing the conformational state of a protein by MALDI MS. Arrows indicate exposed sites susceptible to proteolytic cleavage.

METHODS AND MATERIALS

Trypsin digests of calmodulin (CaM) were performed on trypsin-activated Kratos MALDI sample plates (Intrinsic Bioprobes, Tempe, AZ)[1]. Aliquots of 0.5 μl of a 20 pmol solution of CaM (in 20 mM NH_4HCO_3 containing EDTA or $CaCO_3$) were applied to the sample plate and allowed to digest at room temperature for 5 minutes following the addition of 0.5 μl of sinapinic acid matrix (saturated in 50/50 ACN/0.1%TFA). The sample-matrix mixture was allowed to dry prior to mass analysis. Digests of Methylesterase CheB (CheB) and the phosphorylated form CheB-P were performed in solution (20 mM NH_4HCO_3 and 2 mM $MgCl_2$) with an enzyme to substrate ratio of 1:100 (w/w). In addition, the digest solution of CheB-P contained 50 mM ammonium phosphoramidate. Aliquots of 0.5 μl were taken after 5 minutes of digestion, placed on the MALDI target, mixed with an equal volume of sinapinic acid and allowed to dry prior to mass analysis. All MALDI mass spectra were generated on a Kratos KOMPACT PROBE.

RESULTS AND DISCUSSION

Limited proteolysis experiments of the 17kDa (148 residues) calcium binding protein, calmodulin (CaM), are used to demonstrate the ability of this method to recognize difference in tertiary structure[2]. CaM is a well-studied regulatory protein present in most eukaryotic cells and is known to undergo conformational changes upon binding calcium. The protein contains 4 calcium-binding sites: two in the N-terminal domain and two in the C-terminal domain, both of which are separated by an alpha helix composed of residues 65-92[3]. Peptide mass mapping of CaM in the presence and absence of calcium resulted in two different mass maps (Figure 2).

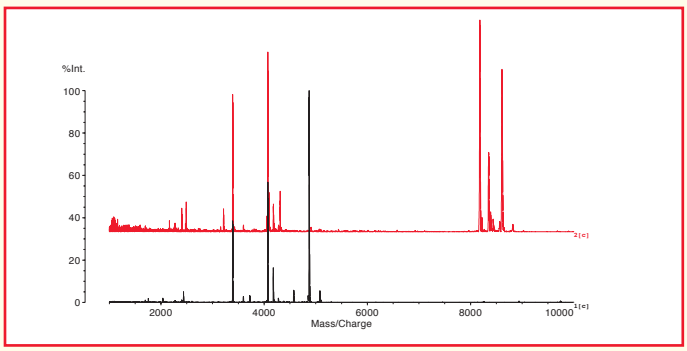

Figure 2. MALDI MS analysis of CaM digests in the presence (bottom) and in the absence (top) of calcium. Differences in the peptide fragments produced in each digest are observed in the spectra.

Fragments resulting from cleavages in the central region of the molecule (specifically, residues 74, 75, and 76) are present in the calcium-free environment. This observation is consistent with the calcium-dependent activation of CaM which proposes (based on x-ray analysis) that upon calcium-binding, CaM undergoes a conformational change in which the central helix acts as a flexible "tether" bringing the two globular domains together to, in effect, hug the target protein[4,5]. In the calcium-free form the protein is said to be more flexible and, as it follows, the results indicate that such flexibility makes CaM more susceptible to digestion. On the other hand, the calcium bound form, a more rigid structure, is observed to be less susceptible to digestion. The mass maps (Figure 2) indicate that the protein has undergone a tertiary structural change in the presence of calcium and in doing so has hindered the accessibility of the enzyme to the central helical region, consistent with the proposed mechanism of activation. Figure 3 shows the sequence coverage in the presence and in the absence of calcium. Of particular note is the difference in the number of cleavages in the central helical region, residues 65-92 (circled).

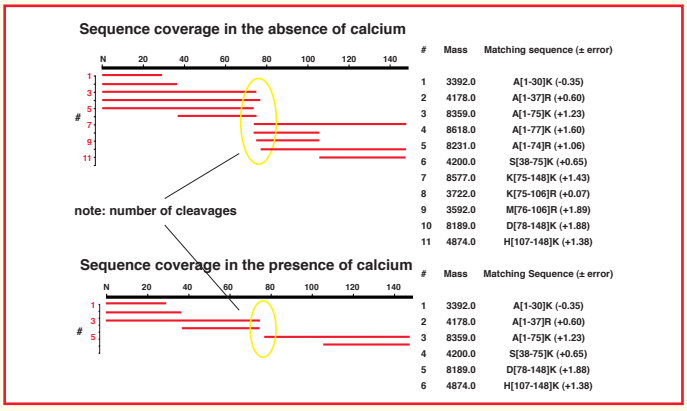

Figure 3. Sequence coverage maps in the presence and absence of calcium after 5 minutes digestion with trypsin.

To further illustrate the utility of the peptide mass mapping approach as a method of identifying differences in protein conformation, digest of the bacterial chemotaxis protein Methylesterase CheB (CheB) in its phosphorylated and unphosphorylated states were performed.

CheB similar to other response regulatory proteins consists of two domains: an N-terminal domain also called the regulatory domain and a C-terminal domain, called the catalytic or effector domain, separated by a linker region. The Asp56 residue, upon phosphorylation, is thought to relieve the inhibition of the catalytic activity of the C-terminal domain. The N-terminal domain is believed to act as both an inhibitor and a stimulator of the C-terminal domain.

The orientation of the two domains, relative to one another, and the effect of this orientation on the activity of the catalytic domain are important questions to those studying CheB and other response regulator proteins. With a half-life of 1 second the phosphorylated form (CheB-P) has not yet been isolated and therefore no x-ray structure has been solved.

In an effort to understand the phosphorylation-dependent activation of CheB, trypsin digests of CheB in the presence and absence of phosphoramidate were performed (phosphoramidate specifically phosphorylated Asp56, the residue responsible for the phospho-activation of CheB). Phosphoramidate was added to the reaction mixture in an excess of a 1000-fold in order to maintain the presence of the phosphorylated form of CheB throughout the experiment. The resulting mass spectra indicate a phosphorylation-dependent difference in the tertiary structure of the protein (Figure 4). Similar to CaM, the major differences in the digestion patterns of the two forms of the protein are the presence of peptides resulting from cleavages in the linker region. Fragments 14836 and 16272 represented only in the mass spectrum of CheB-P, correspond to sequences in the linker region. Upon CheB activation, this region becomes more susceptible to proteolytic cleavage, an indication of a change in the tertiary structure of the protein. Figure 5 shows the sequence coverage map for CheB and CheB-P digests.

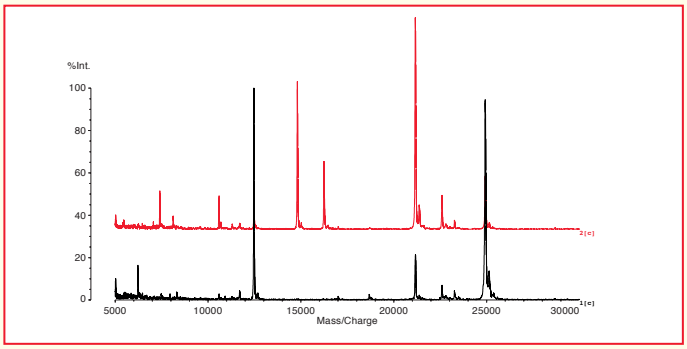

Figure 4. MALDI MS analysis of trypsin digests of CheB-P (top) and CheB (bottom).

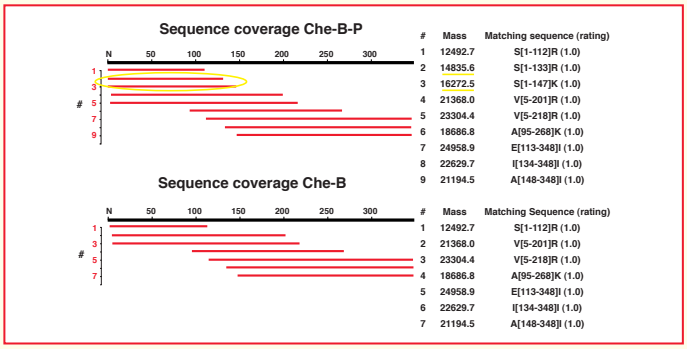

Figure 5. Sequence coverage map for CheB and CheB-P digests.

CONCLUSIONS

Peptide mass mapping may be used to recognize conformational differences (i.e. changes in tertiary structure) in regulatory proteins brought on by the interaction with effectors (e.g. interaction with calcium and covalent modification such as phosphorylation). The observation of qualitative differences when comparing spectra resulting from protein digestion in the absence and in the presence of an effector makes it possible not only to recognize difference in conformational states but to identify which regions of the protein were most affected by the conformational change.

BENEFITS

MALDI MS data complements 3-D structural information obtained by X-ray crystallography and NMR. Proteins and enzymes that are undergoing conformational changes cannot be readily captured in all states that allow an understanding of their functionality. The speed with which MALDI MS can be used for analysis of enzyme reactions, typically only a few minutes for proteases, allows important information to be determined for rapidly changing structural processes. Virtual real time analysis can be performed due to:

- rapid sample preparation and introduction into the mass spectrometer
- relatively high tolerance of MALDI MS to salts and buffers - no sample clean up required prior to mass analysis
- complex mixtures are observed in a single spectrum as only singly charged ions are detected
- with the KOMPACT systems, automated data acquisition means that spectra can be generated easily

The approach described here can be applied to a diverse range of protein systems, whether in isolation or in large complexes, such as viruses, to provide important structural information. Protein biochemists, molecular biologists or production scientists with an interest in correct folding of recombinant proteins will find this an invaluable application.

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