

## Probing Antibody–Antigen Interactions by Mass Spectrometry

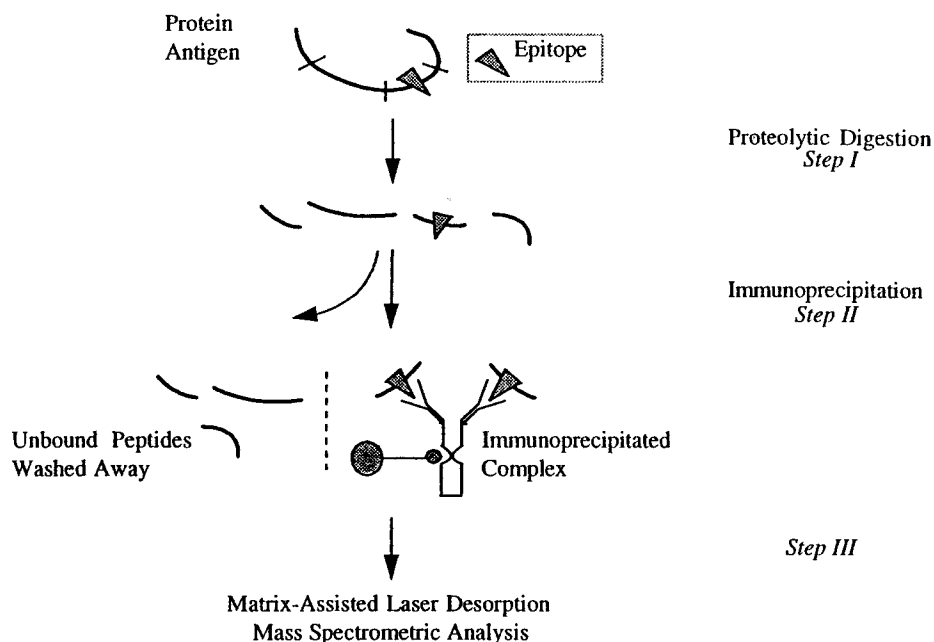
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### 1. Introduction

Techniques that have been used for antigenic site mapping of linear epitopes in proteins include binding assays of protein components produced by synthetic chemistry (1,2) or by recombinant gene expression (3,4). More recently, epitope localization has been achieved through the use of synthetic and bacteriophage peptide libraries (5–9). Although effective, these methods can be costly and time-consuming. A different approach to antigenic site mapping has been reported by Suckau (10), who compared the pattern of proteolytic digestion of free peptide antigen with the pattern produced from the antigen bound to an antibody. Alternatively, these workers subjected the peptide to proteolytic digestion and identified products that bound specifically to the immobilized antibody. In both cases, the peptides of interest were identified by  $^{252}\text{Cf}$  plasma desorption mass spectrometry. Recently, another approach, termed affinity-directed mass spectrometry (11) has been reported by us and others (12–16) for probing antigen–antibody interactions.

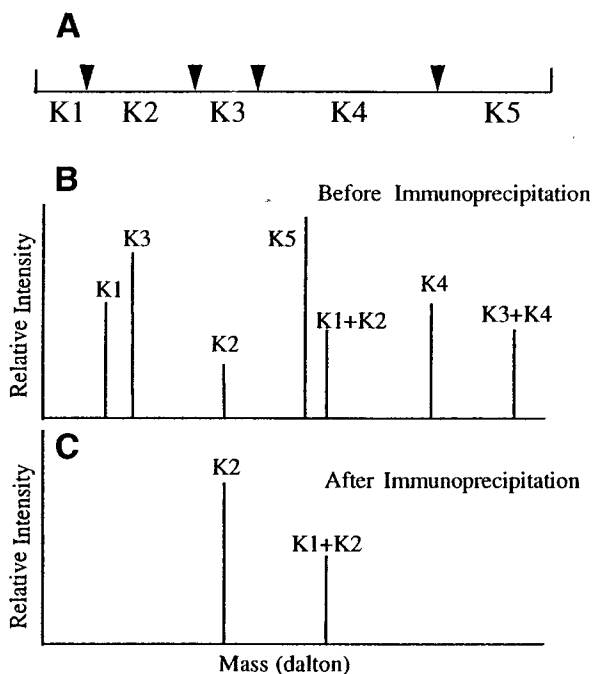
The basis of affinity-directed mass spectrometry is the use of direct molecular mass readout from the immune complex to determine the specific component of the protein antigen that interacts with the antibody. The strategy (12) is shown in Fig. 1. In the first step, a set of peptide fragments is produced by enzymatic digestion of the intact protein. Proteases with known specificity are used so that the sites of cleavage can be predicted and the resulting peptides readily identified by accurate mass measurement by matrix-assisted laser desorption mass spectrometry (MALDI-MS). This step provides an easy method for generating a set of peptide fragments that span the sequence of the protein. Parallel digestion of the protein by two or more proteolytic enzymes with dif-

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**Fig. 1.** Strategy for defining binding sites in a protein that interacts specifically with an MAb (*see text* for details) The antibody is represented by the Y-shaped symbol and protein G plus protein A agarose, by two circles connected by a line. The molecular mass of the peptide in the immune complex is determined by matrix-assisted laser desorption time-of-flight mass spectrometry (17). Under conditions of the mass spectrometric analysis, the epitope-containing peptide dissociates from the antibody and protein G plus protein A agarose and is measured directly as the isolated peptide.

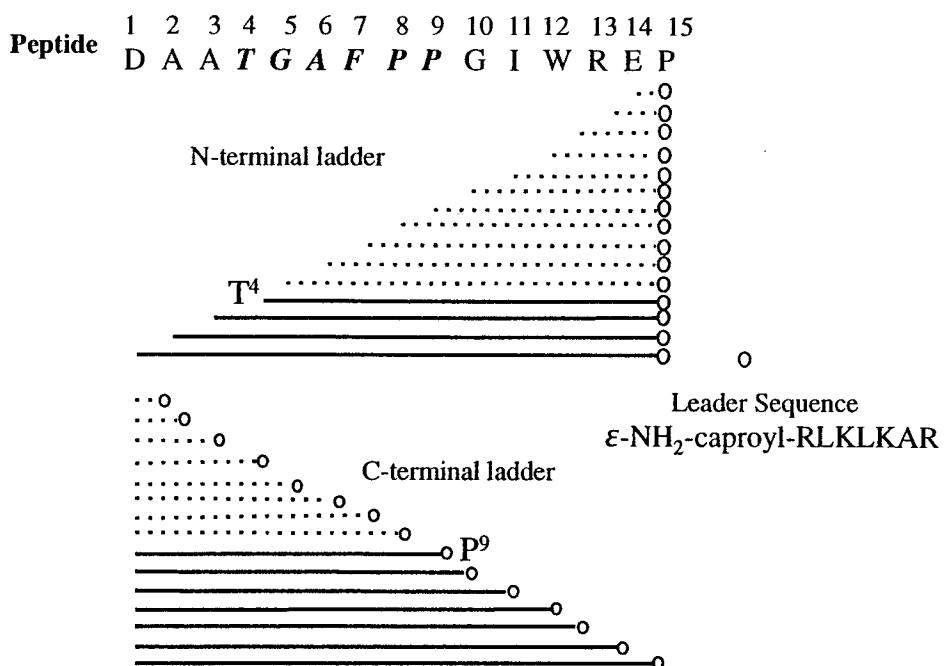
ferent specificities enables rapid production of overlapping sets of protein-spanning peptides. In the second step, the component peptides that contain the binding region of interest are affinity-selected by the immobilized antibody (epitope-containing peptides bind to the antibody and are retained, whereas the remainder are washed away). In the third step, the masses of the affinity-selected peptide fragments are accurately determined directly from the immune complex by MALDI-MS (*see Note 1*). Peptides that are specifically bound to the antibody are identified from their accurately measured molecular masses and a knowledge of the potential digestion sites in the protein antigen. The region of the protein involved in antibody binding is deduced from the sequences of the peptides that are affinity-selected by the antibody. Comparison of these sequences identifies a region of common sequence that contains a dominant component of the binding epitope (Fig. 2). The method uses affinity



**Fig. 2.** A schematic illustration of affinity-directed mass spectrometry for epitope mapping. (A) The linear sequence of a hypothetical antigen protein is represented by a solid line. The antigen protein produces seven peptide fragments after digestion by a protease, whose digestion specificity is indicated by arrows above the lines. Mass spectrometric readout of digested antigen protein before (B) and after (C) immunoprecipitation. After immunoprecipitation, only two peptide fragments (K2 and K2 + K3) are identified in the immunoprecipitated complex, which indicated that the antigenic site is located inside the region K2. The position of each peak in the X-axis of the diagram represents the molecular mass of each component peptide. The height of the peak on the Y-axis represents the relative intensity of each peptide ion species, which depends on the concentration and mass spectrometric ion response of the peptide.

purification in combination with mass spectrometry, and is termed affinity-directed mass spectrometry. The steps outlined above provide low-resolution definition of the binding epitope. The precise boundaries of the binding epitope are determined by affinity-directed mass spectrometric analysis (13,16) of sets of synthetic peptide ladders that span the binding region (Fig. 3).

Proteolytic digestion and affinity-directed mass spectrometry can be used to determine the approximate location of a continuous component of a binding epitope rapidly within a protein ligand. If it is desired to explore the binding of several antibodies against a single protein, the immunoprecipitation step can



**Fig. 3.** Precise determination of boundary residues of antigenic epitope by affinity-directed mass spectrometric analysis of synthetic ladder peptides. The scheme illustrates design and analysis of N-terminal ladder (truncated from the N-terminal) and C-terminal ladder (truncated from the C-terminal) of a hypothetical antigenic peptide (DAATGAFPPGIWREP). The length of the line is proportional to the number of amino acid residues in the peptide. The elliptical symbol designates the leader peptide, ε-NH<sub>2</sub>-caproyl-RLKLR. After immunoprecipitation of the N- and C-terminal ladder peptides with the MAb, only certain components of the ladder mixtures are identified to bind to the antibody. The bold lines designate peptides that bind to the antibody and dashed lines designate peptides that do not bind to the antibody. Inspection of binding properties of peptide ladders shows that T<sup>4</sup> and P<sup>9</sup> are the boundary residues of the epitope. Therefore, residues T<sup>4</sup>GAFPP<sup>9</sup> are required for tight binding to the antibody.

be conveniently carried out in parallel with the set of antibodies. The rapidity of the mass spectrometric analysis (typically only a few minutes/spectrum) allows the approximate location to be determined for several such epitopes in a single day (12). Synthesis and affinity-directed mass spectrometric analysis of peptide ladders containing up to 20 amino acids can be achieved in little more time (1–2 d) than is required to make a single peptide (13,16). Hence, the present approach allows the precise definition of a linear binding epitope for a specific antibody to a short stretch of protein (typically, 6–20 residues,

depending on the available proteolytic digestion sites) in a single day and more accurate definition within 1 wk. For appropriate applications, the procedure should be faster than or competitive with the current approaches. It differs from library-based approaches in that (1) only the natural sequence is explored in the search for a binding epitope and (2) long epitopes can be investigated with little additional effort compared to short epitopes.

We have successfully applied affinity-directed mass spectrometry for epitope mapping to several monoclonal antibodies (MAbs) with binding affinities in the range  $10^{-6}$ – $10^{-9}M$ , including antimelittin MAb #83144 (12), antiglucagon like peptide-1 7-37 MAb #26.1 (12), antihuman basic fibroblast growth factor MAb #11.1 (13,16), and anti-Ad-2 MAb Dave-1 (unpublished results). The results of these experiments demonstrate that the present method should have quite general applicability to the definition of linear epitopes.

## 2. Materials

1. Sequencing-grade endoproteases (e.g., endoprotease Lys C, endoprotease Asp-N, and trypsin) and protease inhibitor Pefabloc Sc (Boehringer Mannheim Biochemical, Indianapolis, IN).
2. Protein G plus/A agarose (Oncogene Science, Uniondale, NY; *see* Note 2).
3.  $\alpha$ -cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO).
4. TSO buffer: 75 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.5% *n*-octylglucoside.
5. TSM buffer: 10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM  $\beta$ -mercaptoethanol.
6. MAbs were purified by protein G plus protein A agarose chromatography (Oncogene Science).
7. Digestion buffers: 50 mM sodium phosphate, pH 8.0, for endoprotease Asp-N; 50 mM Tris-HCl, 1 mM EDTA, pH 8.0, for endoprotease Lys-C.
8. MALDI-MS was carried out in our laboratory with a laser desorption time-of-flight instrument constructed at the Rockefeller University (17,18). Any commercial instrument having similar specifications can also be used.

## 3. Method

### 3.1. Digestion of Proteins

1. Dissolve a 1:30 ratio (w/w) of protease and antigen protein in an appropriate buffer using a protein concentration between 10 and 20  $\mu M$ .
2. Incubate the resulting solution at 37°C for 2 h.
3. Terminate the digestion by adding 1/10 vol of 10 mM Pefablock Sc solution (25°C for 10 min), followed by heating at 90°C for 15 min to inactivate the protease.

### 3.2. Immunoprecipitation (19)

1. Mix an MAb (2–10  $\mu g$ ) and digested protein (20–100 pmol) in TSO buffer (*see* Note 3).
2. After 2 h of incubation at 4°C with gentle stirring, add 2–3  $\mu L$  protein G plus/A agarose to the solution, and incubate for another 0.5–1 h at 4°C. Collect the aga-

rose beads by carefully aspirating the supernatant after centrifuging the solution. Wash the beads three times with TSO buffer and then three times with TSM buffer (*see* Note 4).

### 3.3. Mass Spectrometry

1. To the washed beads, add 4  $\mu\text{L}$  of a saturated matrix solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (*see* Note 5) in 1% aqueous TFA:ACN (2:1) together with an appropriate amount of standard peptide and mix.
2. Measure the molecular masses of the binding peptides: Load 1–2  $\mu\text{L}$  of the matrix–agarose mixture onto the probe tip (*see* Note 6) and dry at room temperature with a stream of air. The mass spectra are collected by adding individual spectra obtained from a large number (50–200) of laser shots to improve the statistics. Spectra can be calibrated either externally or internally using standard peptides.

### 3.4. Synthesis of Peptide Ladders

Solid-phase peptide synthesis is carried out manually as described (20).

1. To synthesize the N-terminal peptide ladder pool, remove an equal portion of peptide resin from the reaction vessel after the addition of each amino acid residue.
2. Mix the resulting peptide resin samples, deprotect, and then subject to HF cleavage. The resulting peptides are used for analysis without further purification.
3. To synthesize the C-terminal ladder peptides, add an equal portion of resin containing the leader peptide,  $\epsilon$ -NH<sub>2</sub>-caproyl-RLKLR (*see* Note 7) after each cycle of the synthesis. The mixed peptide–resin product contains peptides of all possible lengths from the C-terminal amino acid residue.
4. Deprotect and cleave with HF to produce the C-terminal peptide ladder pool.

### 3.5. Affinity-Directed Mass Spectrometric Analysis of Peptide Ladders

1. Immunoprecipitate the N-terminal ladder peptide pool with the MAb (Section 2.).
2. Identify binding peptides by mass spectrometry from the immunoprecipitated complex (Section 3.). The profile of binding and nonbinding peptides provides enough information to define the N-terminal boundary residue of the epitope.
3. The C-terminal boundary residue of the epitope is determined similarly (Fig. 3).

## 4. Notes

1. MALDI-MS is an analytical tool for measuring the molecular masses of peptides and proteins (17,18). The technique allows the accurate (better than 0.1%), rapid (minutes), and sensitive (<1 pmol) determination of the molecular masses of components of complex mixtures of peptides without prior separation. MALDI-MS is finding wide use for the rapid characterization of proteins, and especially the definition of posttranslational modifications and mutations.

2. Protein A agarose and protein G agarose have different binding affinities to immunoglobulins, depending on species and subclasses of the antibodies (21). Protein G plus protein A agarose provides superior performance for binding of most immunoglobulins.
3. A detergent, *n*-octylglucoside is used in the immunoprecipitation buffer (TSO) to reduce nonspecific binding of peptides to the antibody. This is important because nonspecific binding can be a serious problem when mapping antigenic sites of large proteins. SDS and Triton should not be used in the immunoprecipitation, because these detergents suppress the mass spectrometric response.
4. When peaks of nonspecific binding peptides appear in mass spectrum, they can be identified by either comparing profiles of antibody-binding peptides among different digestions or using harsher conditions (more detergents) to wash away nonspecific binding peptides in the immunoprecipitation step.
5. The matrix compound,  $\alpha$ -cyano-4-hydroxycinnamic acid (22) is used to assist conversion of peptides from the solid phase into the gas phase. In addition, the matrix facilitates ionization of the desorbed peptides (18).
6. The antibody-antigen complex together with the protein G plus protein A agarose is loaded directly onto the mass spectrometer probe. The binding peptides will dissociate from the immune complex during mixing with the acidified matrix solution and/or during mass spectrometric analysis.
7. Basic amino acid residues, such as arginine, lysine, and histidine, in a peptide increase the mass spectrometric response of the peptide. To ensure good and uniform mass spectrometric response for the different components of the peptide ladders, a basic leader peptide  $\epsilon$ -NH<sub>2</sub>-caproyl-RLKLR is incorporated at the C-terminal of each peptide. The spacer residue  $\epsilon$ -NH<sub>2</sub> caproic acid is included in the ladder to prevent possible ambiguities that could arise at the junction between the epitope and the ladder sequence.

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