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SPECIAL FEATURE: PERSPECTIVE

Determination of Protein–Protein Interactions by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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A number of different procedures have been developed for use with matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) for the analysis of non-covalent protein–protein complexes. These include use of specific matrix and laser combinations, accumulation of 'first shot' spectra, modification of pH and solvent conditions during sample preparation and use of cross-linking agents to attach the monomers covalently to each other in the complex. The results have shown the techniques to be effective with some but not all complexes, although cross-linking is the most successful. The physical and chemical nature of the complex is critical and therefore a diversity of approaches is recommended for such studies. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: protein–protein interactions; matrix-assisted laser desorption/ionization mass spectrometry

INTRODUCTION

Mass spectrometry has been used to study the structure of peptides and proteins for many years and has benefited from many technological advances during this time. The emergence of desorption/ionization techniques such as plasma desorption and fast atom bombardment in the late 1970s and early 1980s and, more recently, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry has greatly amplified the capabilities of mass spectrometry in this area. Although mass spectrometry has previously been employed primarily for sequence analysis, it is clear that it can also play a role in the measurement of higher order structures.

The study of protein–protein and protein–ligand interactions by mass spectrometry has seen a great deal of activity over the last 10 years. Although much of this work has been done by ESI, and indeed is preferred by many practitioners, MALDI has been used for very high molecular mass complexes and for samples that have proven difficult to analyze by ESI. One of the major advantages of ESI is that the sample may be introduced into the instrument in an aqueous medium, i.e. it does not require the addition of a matrix material. Several more recent papers that describe or review the use of ESI for the study of protein–ligand interactions are recommended.[1–5] In addition, this field will be the subject of a Special Feature article in this journal in upcoming months.

This Perspective article will focus on the use of MALDI to study one particular feature of protein structure, the quaternary structure that results from the non-covalent association of monomeric protein chains to form multi-subunit complexes. The different types of protein structure and the approaches associated with MALDI that are used to study the stoichiometry of protein complexes are described. These include use of several types of laser–matrix combinations, data obtained from 'first shot' experiments and varying the sample preparation conditions to optimize the measurement of the multimeric complex by mass spectrometry. Finally, the use of chemical reagents to cross-link the chains within the complex is described.

PROTEIN STRUCTURE

Proteins are essential components of cells and are involved in many cellular processes, including mechanical transport, enzyme catalysis, differentiation and growth and cell–cell recognition. The interactions of proteins both within the polypeptide chain and with other molecules is mediated by a number of forces that include electrostatic, hydrophobic, hydrogen bonding and van der Waal's interactions. Essentially these forces help determine the structure and behaviour of the polypeptide chains in solution.[6–9] Protein structure is generally classified at four levels, as follows.

1. Primary structure refers to the amino acid sequence.

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2. Secondary structure involves the interaction of amino acids within the chain to form hydrogen-bonded structures such as α-helices, β-bends, β-sheets and random coils. β-Sheet and α-helical regions are common in proteins. The α-helix is usually a right-handed helix and has 3.6 amino acids per turn with side-chains extending outward in a helical array. This allows the formation of hydrogen bonds between the carbonyl groups and the amino groups of the same peptide backbone, which results in an extremely stable structure. β-Sheets form hydrogen bonds between the carbonyl groups and the amino groups either within regions of a protein or between protein molecules and thus form planar arrays or 'sheets' rather than coiled structures.
3. Tertiary structure is the three-dimensional structure formed by the interaction of a single polypeptide chain or covalently linked chains and is often categorized as either fibrous or globular. Fibrous structures, such as keratin, collagen and elastin, are involved in structural components of animal cells and tissues and are generally insoluble in water. Globular proteins are generally water soluble and comprise a large class of proteins. In an aqueous environment, the nature of the tertiary structure is driven by the energetic advantage of minimizing contact of hydrophobic regions with water, but ionic interactions and disulfide bonds also contribute to the stability of the folded structure. The entropy (ΔS) increase that results from burying hydrophobic regions in the interior of the protein, combined with the decreased enthalpy (ΔH) due to the interactions between groups within the folded structure results in a negative free energy (ΔG) value to yield a thermodynamically favorable process.
4. Quaternary structure is the interaction of non-covalently bound monomeric protein subunits to form oligomers. Such complexes are common in eukaryotic cells and are involved in many critical cellular processes. The monomeric protein subunits need not be identical. Forces that direct the interactions between the subunits are the same as those involved in the formation of secondary and tertiary structure. While hydrophobic interactions are thought to make the greatest contribution to subunit interactions, hydrogen bonds and salt linkages also contribute to the specificity of subunit interaction.[10] Quaternary structure is divided into a number of categories including the number of subunits that interact (stoichiometry), whether the subunits are identical or not, the arrangement of the subunits in space and the type of contact the subunits have with each other.

The various bonds and forces giving rise to protein structure are critical to protein stability and activity. Disruption of these forces by such means as changes in pH, temperature or pressure and ionic strength and addition of hydrogen bond-disrupting agents such as urea, addition of reducing agents or changes in solvent can cause partial or complete protein denaturation. Once this occurs, the activity of the protein is often lost and, generally, only partial activity is regained if the conditions are reversed. A complication for the measurement of quaternary structure is that many proteins can aggregate or associate through non-specific binding, a process that is concentration dependent. These complexes are artifacts in that they are not of physiological significance, i.e. they do not have the structure or activity of the biologically competent multisubunit complex.

DETERMINATION OF STOICHIOMETRY OF COMPLEXES WITH MALDI/MS

MALDI/MS has been an extremely powerful tool for determining the molecular masses of proteins and also other aspects of protein structure.[11] Analytes of interest are mixed with a matrix that absorbs energy at the wavelength of the laser. For most commercial instruments, a nitrogen laser is used together with a low molecular mass organic matrix (e.g. dihydroxybenzoic acid, cinnamic acid analogs). Most of the matrices used are highly acidic and require organic solvent for dissolution.

In general, most protein oligomers dissociate when analyzed by MALDI/MS and it was concluded early in the development that this technique was too energetic to allow non-covalent protein complexes to remain intact throughout sample preparation and analysis. Nevertheless, there appear to be a number of exceptions. The first determination of the number of protein subunits by MALDI/MS was accomplished with a quadrupled Nd:YAG laser ($\lambda = 266$ nm) with nicotinic acid as the matrix. Streptavidin (*Streptomyces avidinii*) was desorbed from a nicotinic acid matrix dissolved in 10% ethanol in water.[12] The base peak in the spectrum (accumulated from 10 single-shot spectra) is the monomer with additional peaks recorded for dimer (80% monomer intensity), trimer (30% monomer intensity), and tetramer (60% monomer intensity) as shown in Fig. 1. On addition of 50% ethanol to the sample, MALDI/MS analysis showed the monomer to be the only peak in the spectrum. Glucose isomerase[13] and jack bean urease[14] were also analyzed using a quadrupled Nd:YAG laser ($\lambda = 266$ nm), with nicotinic acid as the matrix, to determine the correct number of subunits in the oligomer.

Although most non-covalent protein–protein complexes dissociate under methodological conditions employed for samples analyzed using the nitrogen laser

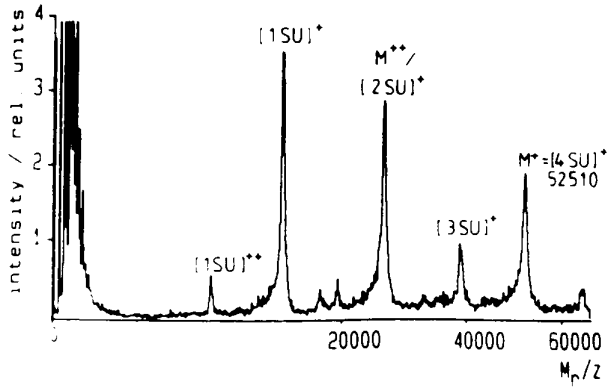

Figure 1. MALDI mass spectra of strepavidin (from *Streptomyces avidinii*), nicotinc acid matrix (10% ethanolic solution), 266 nm accumulated from 10 single-shot spectra.[12] SU = subunit. Reproduced with permission.

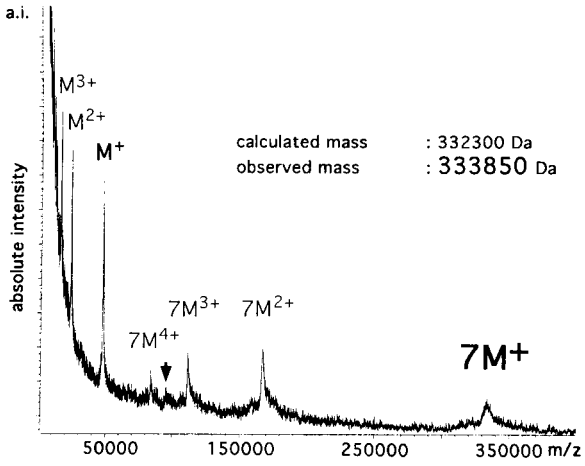

Figure 2. MALDI mass spectrum of aerolysin heptamer.[15] Measured molecular mass 333 850 Da. Less than 500 fmol loaded on the target in sinapinic acid, 337 nm, monomer: 7M, heptamer. The arrow indicates a small peak corresponding to traces of aerrolysin dimers. Reproduced with permission.

and sinapinic acid matrix, several multisubunit complexes have been recorded. For example, aerolysin, a membrane-associated protein that forms pores, is an extremely stable multi-subunit complex that survives treatment with SDS, pH extremes, guanidine hydrochloride, reducing agents or heat. However, it can be dissociated upon incubation in 70% formic acid for 30 min at room temperature. Recent work[15] reported the MALDI/MS analysis of this protein (2 μM monomer concentration) in sinapinic acid (13 mg ml^{-1} in 50:50 0.1% TFA–acetonitrile) using a nitrogen laser. The monomer (*m/z* 47 480) was the base peak and the heptamer (*m/z* 333 850) was 20% of that intensity, with other peaks present being the doubly and triply charged ions of each species (Fig. 2). The peak for the heptamer was recorded only in the concentration range 0.5–1.5 pmol μl^{-1} protein at threshold irradiance with matrix-to-analyte ratios in the range 20 000:1–50 000:1.

FIRST SHOT ANALYSIS

Some recent work on the determination of protein stoichiometry utilized a method termed 'first-shot' MALDI/MS analysis.[16] First laser shots on parts of the sample not previously irradiated are collected and averaged to give spectra of the intact oligomer. Omp F porin protein from *E. coli* is a homotrimer that forms a pore allowing transport of small hydrophilic molecules (<600 u) across the cell membrane. Mass analysis of porin with ferulic acid matrix in tetrahydrofuran (THF) using a tripled Nd:YAG laser ($\lambda = 355$ nm) showed that the most intense peak corresponded to the trimer (*m/z* 111 250) (Fig. 3). This result was obtained only upon irradiating the sample at threshold irradiance with one shot at each individual spot and summing only the first shots (average of 10 first shots at different spots) using a matrix-to-analyte ratio of approximately 40 000:1–50 000:1. Comparable results were obtained using nicotinic acid in water with a quadrupled Nd:YAG laser ($\lambda = 266$ nm) by summing the first shots (10 averaged) on different areas of the prepared

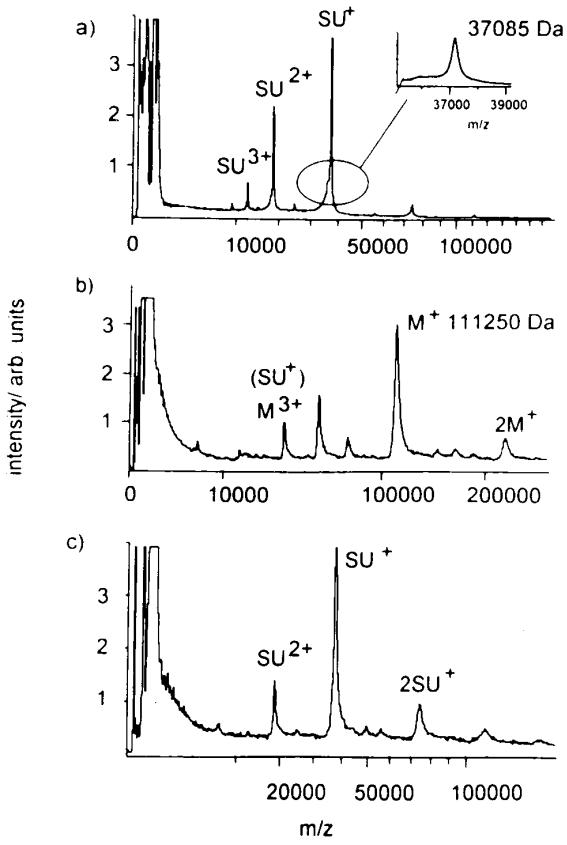

Figure 3. UV–MALDI mass spectra of porin.[16] Wavelength 355 nm. Sum of 10 single spectra each. (a) Matrix: DHBs. (b) Matrix: ferulic acid in THF. Sum of first shots on a given spot. (c) Matrix: ferulic acid in THF. Sum of second and following shots on a given spot.

sample. Investigation of other solvents for the matrix (acetonitrile, acetonitrile–0.1% trifluoroacetic acid (TFA)) gave the monomer as the base peak in the spectra using ferulic acid as the matrix. The authors stated, 'It is important to note that a prominent signal for the intact quaternary protein (porin trimer) can only be obtained from the first laser shot on a not-yet-irradiated sample spot.' Matrix crystal morphology was also found to be important for the detection of the intact porin trimer with smaller, finer crystals giving better results over larger, feather-like crystals formed when using a different solvent composition.

Different matrices and solvent systems have been investigated in an attempt to optimize protein quaternary structure determination using MALDI/MS.[17] Streptavidin, a homotetramer, was used to test these conditions. Use of a mixed matrix (10 g l^{-1} solutions of 2,5-dihydroxybenzoic acid–2-hydroxy-5-methoxybenzoic acid (9:1, v/v) gave a distribution of monomer, dimer, trimer and tetramer. The monomer was the most intense peak with successive oligomer peaks decreasing in intensity as *m/z* increased. This suggests non-specific protein–protein aggregation, similar to that seen for chicken egg white lysozyme, which is known to self-associate to form a distribution of oligomeric forms.

Additional work with streptavidin showed the tetramer (*m/z* 51 870) to be the most intense peak in the spectrum using 2′,6′-dihydroxyacetophenone (DHAP) as the matrix in acetonitrile (ACN)–TFA.[17] Peaks were also present corresponding in *m/z* values to monomer, dimer

and trimer using the first shot technique (Fig. 4). Employing the same matrix and solvent, but with 25 laser shots on the same sample spot, the monomer was the most intense peak in the spectrum with only a small dimer peak visible. The streptavidin tetramer was detected using (i) ferulic acid in THF and ACN–TFA as solvents, (ii) 2′,4′-DHAP and 2′,6′-DHAP in THF, ethanol and ACN–TFA, (iii) 2′,5′-DHAP in THF and (iv) 2′,4′,6′-trihydroxyacetophenone (THAP) in ACN–TFA. The hydroxyacetophenone matrices were chosen because their acidity was less than that of other matrices. Higher peak intensities with better resolution and reproducibility were obtained using 2′,6′-DHAP in THF or acetonitrile–TFA than other matrices investigated.

Beef liver catalase and yeast alcohol dehydrogenase (yADH) were analyzed using 2′,6′-DHAP–THF with first shot analysis. For yADH, the dimer (*m/z* 73 870) was the most intense peak in the spectrum, with the peak corresponding to the tetramer (*m/z* 147 940) being ~80% of the peak intensity of the monomer. Averaged shots at the same spot gave monomer and dimer peaks of almost equal intensity. Similar results were obtained for beef liver catalase using 2′,6′-DHAP–THF with the averaged first shots giving an intense peak at the *m/z* value corresponding to the tetramer and also intense peaks at the *m/z* values for the monomer and the dimer. Interestingly, no peak appeared at the *m/z* value for the trimer using this matrix combination. Successive shots at the same spot yielded the monomer as the most intense peak in the spectrum for catalase with successively smaller peaks corresponding to the dimer and trimer. The concentration of the protein sample is an important factor in observing the intact quaternary structure. For streptavidin, tetramers were observed for concentrations between 0.05 and 10 g l^{-1} (3.8–770 pmol μl^{-1}) whereas for yADH tetramers were observed only for concentrations between 0.1 and 0.5 g l^{-1} (2.7–13.5 pmol μl^{-1}).

The value of added proteins for internal calibration was assessed with the first shot analysis method.

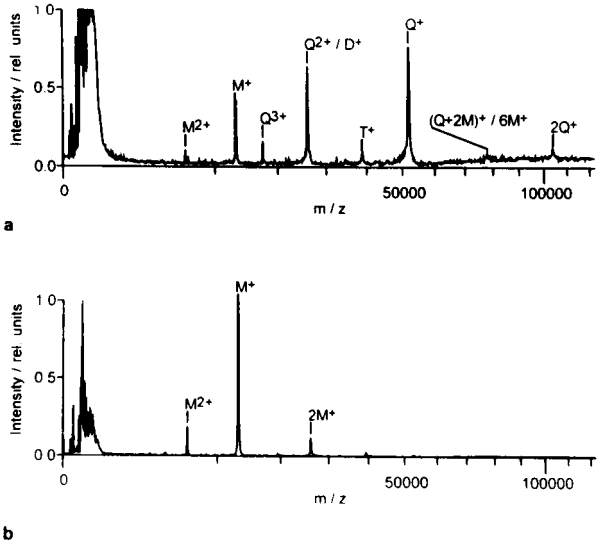

Figure 4. Streptavidin in 2,6-dihydroxyacetophenone–ACN–TFA.[17] $\lambda = 355$ nm. M = monomer; D = dimer; T = trimer; Q = tetramer. (a) First shot only (30 spectra total); (b) 25 shots on the same spot. Reproduced with permission.

MALDI/MS analysis of streptavidin with horse heart myoglobin and subtilisin Carlsberg as internal calibrants caused a 'dramatic' decrease in the intensity of the tetramer peak.[17] The authors attributed this to dissociation effects or competition of the tetramer ion with the calibrant protein ions for total charge in the sample. Additional factors are probably involved because experiments with protein mixtures consisting of both oligomeric and monomeric proteins suggest that even with high concentrations of added proteins, the specific association of oligomeric proteins in a complex is not necessarily disturbed.[18]

EFFECT OF MATRIX CONCENTRATION AND pH

It has been postulated that in many cases oligomeric species are not routinely observed by MALDI/MS because the condition of low pH and high concentrations of organic solvents used to make up most matrix solutions dissociates the protein oligomers. Several investigators have attempted to control the pH of the matrix and/or analyte solution to preserve the intact quaternary structure. Enzyme–substrate complexes were the first samples used to test the effectiveness of pH modification of the matrix and analyte solution for MALDI analysis of protein–protein interactions.[19] Peptides were dissolved in 1 M ammonium citrate (pH 6.0) and enzymes in 3.2 M ammonium sulfate (pH 7.0) at a concentration of 10 pmol μl^{-1}. The matrix, sinapinic acid, was saturated in ethanol–1 M ammonium citrate (1 : 1). The peptide was deposited on the target followed by the enzyme and then the matrix was added immediately to quench the reaction. This mixture was then allowed to dry at room temperature. Three enzyme–substrate complexes were studied: aminopeptidase I and bovine growth hormone-releasing factor (GHRF), trypsin and rat parathyroid hormone fragment (1–34) (RPH) and arylamidase and GHRF.

Mass analysis of aminopeptidase I and GHRF showed ions present at *m/z* values for the enzyme, its substrate and the enzyme–substrate complex.[19] The molecular species for GHRF at *m/z* 5109 was the base peak in the spectrum (Fig. 5) and was accompanied by those for the enzyme at *m/z* 29 971 (13% GHRF intensity) and the complex at *m/z* 35 118 (1.5% GHRF intensity). Ions for the enzyme–substrate complex were absent from the mass spectrum when the matrix was prepared in ethanol–water (1 : 1) (pH < 2).

Similar results were found for the other two enzyme–substrate complexes. The RPH signal at *m/z* 4059 was the base peak in the spectrum, with those for trypsin (*m/z* 23 208) being 32% of the RPH intensity, and for the trypsin–RPH complex (*m/z* 27 243) being 2% of the RPH intensity.[19] Dilution of the trypsin five-fold caused the enzyme–substrate complex to disappear from the mass spectrum. Likewise, the signal intensity for arylamidase (*m/z* 29 798) was 15% of the GHRF intensity and the intensity of the arylamidase–GHRF complex (*m/z* 34 884) was 1% of the GHRF intensity. The use of an acidic matrix also eliminated the peak for the enzyme–substrate complex in the MALDI mass spectrum for both complexes described. To test for non-

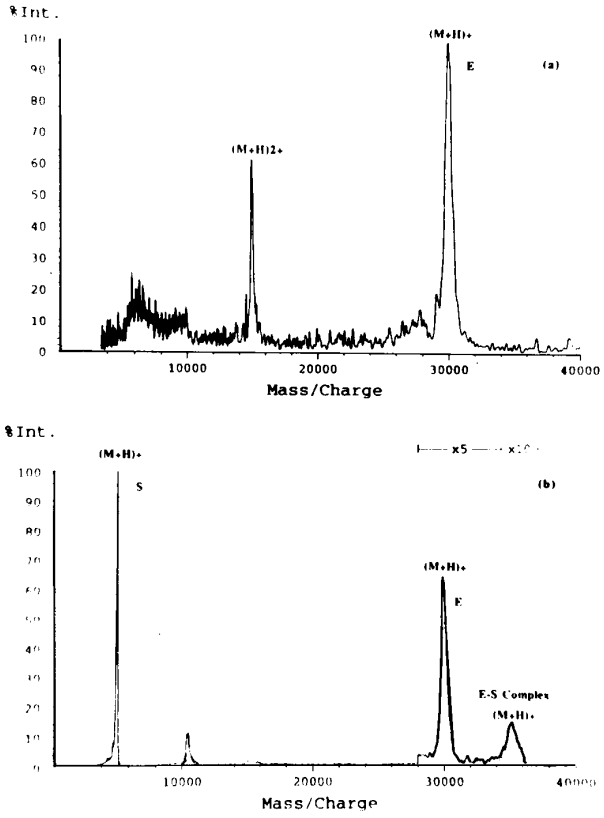

Figure 5. MALDI mass spectrum of (a) enzyme (aminopeptidase 1) and (b) substrate (GH), enzyme (aminopeptidase 1) and enzyme–substrate complex.[19] E, enzyme; S, substrate; sinapinic acid; 337 nm. Reproduced with permission.

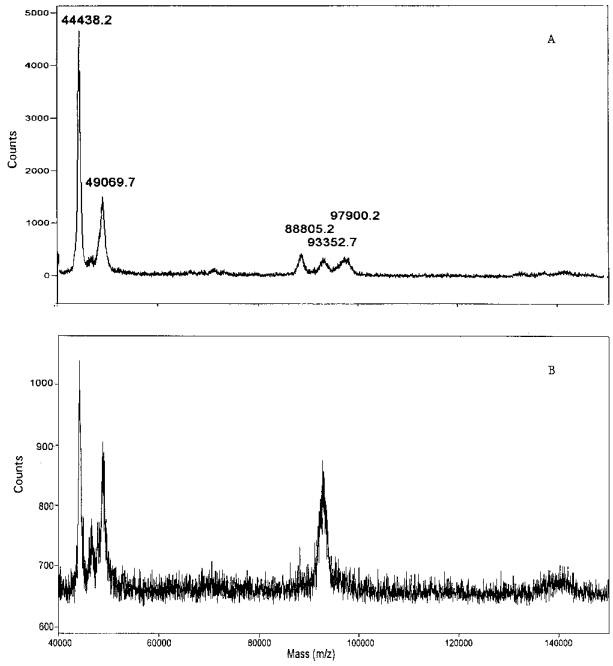

Figure 6. Effect of sample preparation conditions on the MALDI mass spectra of human farnesyl protein transferase.[20] The sinipinic acid matrix was dissolved in (A) ACN–0.1% TFA (70:30) and (B) ACN–0.2 M Bis–Tris (30:70) (pH 7). Reproduced with permission.

specific complex formation in the gas phase, three proteins (GH, PRH and cytochrome *c*) were mixed and analyzed under the same MALDI/MS conditions using both buffered and acidic matrix solutions. The resulting spectra showed no formation of complexes between the three proteins. This is not necessarily conclusive, of course, since the added proteins do not associate with the proteins in the liquid or solid phase, and therefore may not show association in the gas phase under the same conditions.

Human farnesyl protein transferase is a heterodimer consisting of one α- and one β-subunit. Analysis of the mixture using sinapinic acid dissolved in ACN–0.1% TFA (70:30) gave peaks corresponding to each of the subunit monomers (at m/z 44 438 and 49 070) in addition to three low-intensity peaks of about equal intensity corresponding to non-native homodimers (α_2 at m/z 88 805 and β_2 at m/z 97 900) in addition to the native heterodimer ($\alpha\beta$ at m/z 93 353) [Fig. 6(A)].[20] Using sinapinic acid dissolved in ACN–0.2 M Bis–Tris (30:70) (pH 7), a peak corresponding to the heterodimer was seen in the spectrum with an intensity equal to that of the β-subunit and 40% of that of the α-subunit [Fig. 6(B)]. No non-native homodimers were recorded in the spectrum obtained by averaging 100 shots from the same sample spot. Cross-linking of the protein complex in solution with glutaraldehyde, described in the next section, yielded a peak corresponding to the heterodimer that was the base peak in the spectrum.

The specific non-covalent interactions of RNAse S and of leucine zipper dimers using 6-aza-2-thiothymine (ATT) as the matrix have been reported. RNAse S is a non-covalent complex composed of S-peptide and S-protein. At pH 5.5, a peak for the intact RNAse S complex was recorded in the spectrum at m/z 13 630 (Fig. 7)[21] at relatively low intensity compared with the signals for the S-peptide (m/z 2096) and the S-protein (m/z 11 500). At pH 2, the complex was completely dissociated and only the molecular ions for the individual subunits were detected. Results from addition of 0.1% TFA to the sample–matrix preparation followed by drying and subsequent MALDI/MS analysis showed that complete dissociation occurred upon acidification. The analysis of the RNAse S complex was also concentration dependent (15–367 μM). Below 15 μM, no complex was observed in the MALDI mass spectrum, consistent with reported K_d values of $<10^{-8}$–2×10^{-5} M between pH 7 and pH 3. The specificity of the interaction was tested by the addition of angiotensin to the RNAse complex. No non-specific interaction was observed under conditions where the RNAse complex was seen in the spectrum. Dissolving both the matrix and analytes in ammonium hydrogencarbonate, ammonium acetate and ammonium citrate gave the most intense signal for the complex.

Leucine zipper polypeptides were investigated using ATT in buffered solutions.[21] Dimers (m/z 6533) of low intensity (~20% of the monomer) were observed in the MALDI mass spectrum using ATT whereas the use of HCCA in ACN–0.1% TFA showed no dimer peaks in the spectrum. Addition of angiotensin with subsequent analysis by MALDI/MS using ATT indicated that there were no non-specific interactions. Mass analysis of a mixture of two different leucine zipper peptides (A at m/z 3574 and B at m/z 3687) showed dimer formation corresponding to the homodimers (AA and BB at m/z 7147 and 7373, respectively) and to the heterodimer (AB at m/z 7260). MALDI/MS analysis of the *C*-terminal

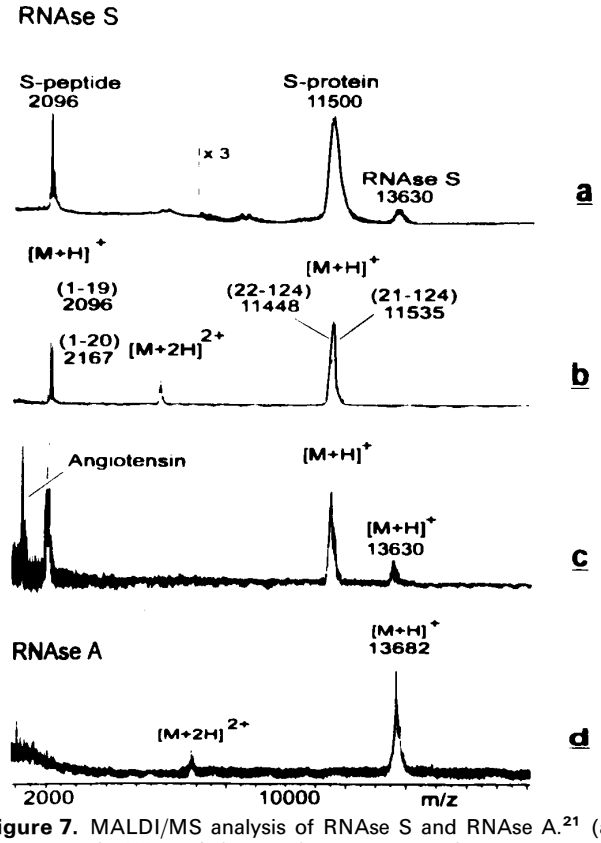

Figure 7. MALDI/MS analysis of RNAse S and RNAse A.[21] (a) Spectrum of RNAase S (147 μM) dissolved in 10 mM ammonium citrate with ATT (5 mg ml^{-1}), dissolved in 10 mM ammonium acetate as matrix, pH 5.5. (b) Spectrum of RNAse S in ACN–0.1% TFA (2:1, v/v) (pH 2) with HCCA as matrix. (c) Spectrum of RNAase S using the sample preparation as in (a) after addition of an equimolar amount of angiofensin. (d) Spectrum of RNAse A (147 μM, prepared as in (a)).

domain of the yeast transcription activator protein, a leucine zipper domain, also showed the formation of the dimer with higher peak intensity compared with that previously seen in this work (monomer to dimer ratio 2:1).

The pH of the sample is an important factor in the detection of non-covalent complexes and changing conditions of pH and ionic strength due to solvent evaporation will affect the complex stability by disrupting inter- and intramolecular forces. For example, the interaction of RNAse S and leucine zippers is dictated primarily by hydrophobic interactions and the lack of organic solvent in the sample preparation may also contribute to the observation of these complexes using this preparation method and ATT as the matrix. The spectra were obtained by averaging up to 100 shots from the same sample spot, and the laser power and the depth of penetration did not appear to have a significant influence on the observation of the complexes.[21]

CHEMICAL CROSS-LINKING

Chemical cross-linking using imido esters has been employed in biochemistry laboratories for many years to determine the stoichiometry of oligomers with analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[22] Recently, chemical cross-linking followed by MALDI/MS analysis with a nitrogen laser and sinapinic acid matrix (saturated in ACN–0.1% TFA (70:30)) has been utilized for this purpose.[23] MALDI/MS analysis of the protein oligomer before cross-linking permitted the determination of the molecular masses of the individual subunits and the compositions, i.e. whether the oligomer was composed of identical or non-identical subunits. A homobifunctional or heterobifunctional cross-linking agent was then added to the protein solution and the stoichiometry of the oligomeric complex was determined.

One of the most generally useful cross-linking agents is glutaraldehyde, a homobifunctional reagent, that reacts primarily with the ε-amino group of lysine to form cross-linking chains of differing lengths. The reaction can be stopped by the addition of $NaBH_4$ or, for MALDI analysis, by the addition of matrix. For example, bovine hemoglobin is a heterotetramer made up of two α-subunits and two β-subunits that forms an $\alpha_2\beta_2$ tetramer. MALDI/MS analysis prior to cross-linking showed the presence of the individual subunits at m/z 15 042 and 15 950 and also the formation of non-specific dimers (α_2, β_2 and $\alpha\beta$) of low abundance as shown in Fig. 8(A).[18] It is assumed in this case that the $\alpha\beta$ dimer is non-specific because it is of the same abundance as the non-physiological dimers at α_2 and β_2. After cross-linking with glutaraldehyde, the MALDI mass spectrum showed the presence of both monomers, a dimer corresponding to only the physiological $\alpha\beta$ form, a small amount of trimer and a tetramer corresponding to the $\alpha_2\beta_2$ tetramer [Fig. 8(B)]. The peaks were shifted higher in mass relative to the native forms owing to the addition of glutaraldehyde as the cross-linker. For the cross-linked tetramer, it was calculated that an average of ~21 glutaraldehyde units per monomer were added to the protein, although from the molecular mass difference it is not possible to determine how many cross-links this represents. To confirm the specificity, the cross-linking reaction was run under conditions which would dissociate the complex (50% methanol) and the resulting spectrum showed only the two monomers again shifted to higher m/z because of the addition of glutaraldehyde.

Recombinant nitric oxide synthase (NOS) catalyzes

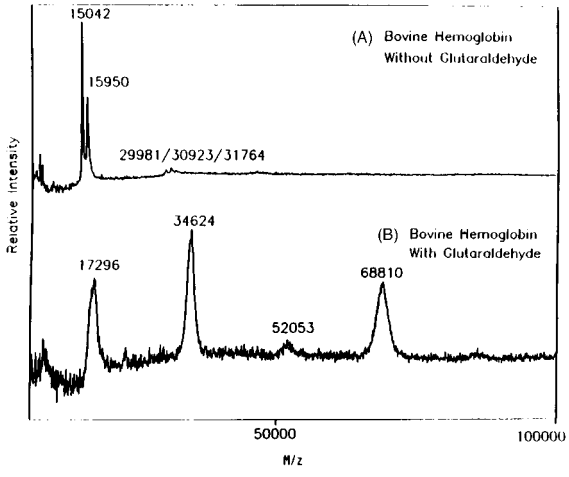

Figure 8. MALDI mass spectra of bovine hemoglobin (A) native complex and (B) native complex after crosslinking with glutaraldehyde.[23] The matrix was sinapinic acid, used with the nitrogen (337 nm) laser.

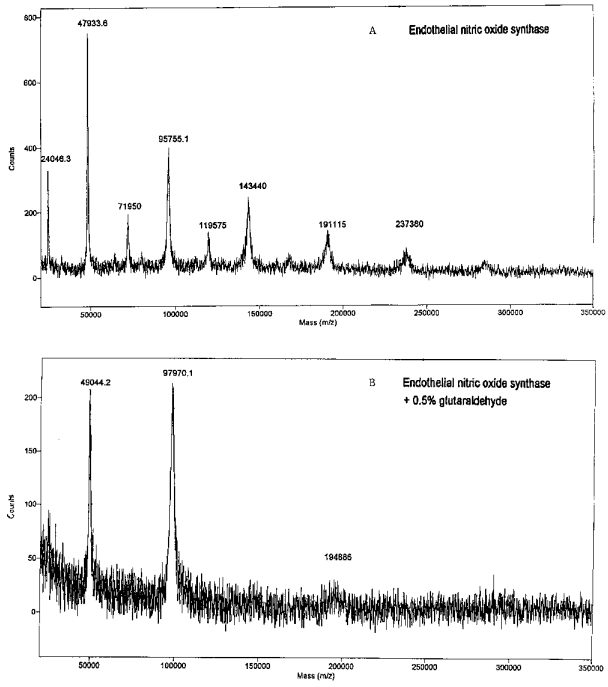

Figure 9. MALDI mass spectra of nitric oxide synthase (A) before and (B) after cross-linking with glutaraldehyde.[24]

the conversion of arginine, oxygen and NADPH into citruline, nitric oxide and $NADP^+$ and is believed to be a dimer. Cross-linking experiments on recombinant human NOS protein with 0.5% glutaraldehyde combined with MALDI/MS analysis provided evidence for the dimeric nature of this non-covalent association.[24] The MALDI mass spectra of the native protein showed an intense $[M + H]^+$ ion signal at m/z 47 934 (Fig. 9), and lower intensity signals corresponding to the dimer and trimer species were also present. Analysis of the cross-linked samples (20 μM solution) showed mainly the presence of the monomeric and dimeric species with ~13 glutaraldehyde units added to the NOS molecule. The heme unit was lost and an ion corresponding to this mass could be seen in the low-mass range.

The combination of glutaraldehyde cross-linking and MALDI/MS analysis has been employed with many protein–protein non-covalent complexes, including dimers (human farnesyl protein transferase, XΨ complex of DNA polymerase III holoenzyme and glucose 6-phosphate dehydrogenase), tetramers (yADH, pyruvate kinase and the β_4 complex of the DNA polymerase III holoenzyme) and a hexamer (bovine glutamate dehydrogenase). Other cross-linking reagents may also be used, such as that reported with heterobifunctional azido cross-linkers to determine the stoichiometry of native forms of proteins.[13]

H–D EXCHANGE

The use of H–D exchange to study protein–protein interactions has not yet been developed using MALDI/MS, although a number of studies have employed this technique in combination with ESI-MS. Regions of the proteins in close contact or that are involved in intermolecular bonding forces should have a different rate of exchange relative to other regions more accessible to the solvent. Future developments in this area may be of considerable value in the study of protein–protein interactions. For a brief review of the use of H–D exchange in the study of protein structure, see the Special Feature by Smith et al.,[6] Probing the Non-covalent Structure of Proteins by Amide Hydrogen Exchange and Mass Spectrometry.[25]

CONCLUSIONS

Non-covalent complexes are of great importance to protein stability, function and regulation in cells and, because of this, a great deal of work has been devoted to determining the nature of protein–protein and protein–ligand interactions. Included in this paper are four methods for MALDI/MS analysis that have been reported in determining protein quaternary structure:

1. Nicotinic acid matrix dissolved in water and using an Nd:YAG laser.
2. First shot analysis where only first shots of the laser on the sample are collected and averaged.
3. Modification of the pH of the matrix and/or sample mixture by dissolution in buffer solutions.
4. Cross-linking the sample in solution with subsequent MALDI/MS analysis of the cross-linked product.

It is obvious that the first three methods only work with certain complexes, i.e. a successful analysis occurs where the methods are compatible with the chemistry of the complex. Cross-linking is much more reliable but, of course, requires some additional preparation and it must be kept in mind that the reagents employed may also alter the native state of the protein.

The advantages of using MALDI/MS for these analyses over more traditional biochemical methods are considerable. The mass accuracy is greater, the time of analysis is significantly shorter and the amount of sample used is much less than in other methods commonly employed in biochemistry laboratories, such as gel electrophoresis. MALDI/MS can be used to determine whether the subunits involved in protein–protein interactions are identical or different, the molecular mass of the individual subunits and the stoichiometry of protein–protein interactions. However, sample preparation for a given complex may not be trivial. Protein concentration, the matrix, matrix-to-analyte concentration, solvents, ionic strength and pH are all important variables that influence the detection of non-covalent protein complexes by MALDI/MS.

Although, overall, MALDI/MS procedures have had some success in measuring multi-subunit complexes, some cautions need to be noted. Simple observation of a low-intensity signal for an oligomer in the mass spectrum is not sufficient evidence that the physiologically active complex has been measured in the gas phase. Several factors should be kept in mind. First, it is known that even those proteins that associate to form physiologically active oligomeric complexes may also aggregate through non-specific binding. These proteins generally produce MALDI mass spectra with the monomer as the base peak and additional peaks of decreasing intensity corresponding to dimers, trimers and other oligomers depending on the matrix used, the sample concentration and the tendency to aggregate.

There are non-physiological oligomers, i.e. oligomers from non-specific associations that are not necessary for biological activity and, indeed, often significantly inhibit activity. Decreasing the laser power to the threshold level and decreasing the concentration of the protein in solution can minimize this phenomenon. For example, dilution of an aggregating species such as chicken egg white lysozyme gives decreasing numbers of these non-specific aggregate peaks in the spectrum. Second, the mass spectrum often contains 'laser-induced' oligomeric artifacts, i.e. generally low-level peaks ($<10\%$ of the monomer) that are a consequence of the ionization process perhaps in combination with some non-specific binding. Recall that MALDI/MS of the $\alpha_2\beta_2$ tetramer of hemoglobin produces not only the physiological $\alpha\beta$ dimer, but also α_2 and β_2 dimers, with the latter two being artifacts of the MALDI process.

The basic problem of relating gas-phase measurements of protein higher order structure with the physiologically competent structure in solution is not a trivial one to solve. The quaternary structure of the multi-subunit complex in solution is dependent not only on the primary structure, but also on the secondary and tertiary structure, which in turn may be significantly influenced by the solvent composition and the presence or absence of salts and impurities. Perturbation of this solution-phase structure in some manner after mixing with matrix, drying and laser desorption is not an unreasonable expectation. It is therefore imperative in such measurements to perform a number of control experiments in an attempt to test the degree to which this structure has been perturbed. Some suggested controls are:

1. Compare the spectra of native and denatured preparations of the protein. Several types of denaturation processes should also be compared, such as use of pH, salt, heat and chemical denaturants. Can oligomers be seen in the spectrum and is the distribution the same among those produced by different denaturation processes?
2. Determine the concentration dependence for observing the quaternary structure in the spectrum.
3. Measure the effect of addition of various concentrations of another protein. Examine the effect using a protein of different and similar isoelectric points and/or hydrophobicity/hydrophilicity, if possible.
4. Measure the effect of different matrices, particularly in changing from acidic to neutral matrices.
5. Examine the first shot spectrum and compare it with multiple-shot (summed) spectra. The first shot analysis may be preferred if the amount of protein is limiting.
6. Measure the quaternary structure by MALDI/MS after treating the complex with cross-linking agents. The effectiveness of these agents depends on the particular residues available in the complex, although we have found dilute concentrations of glutaraldehyde to be effective owing to the large number of lysine residues generally found in hydrophilic proteins. There are, of course, many homo- and hetero-bifunctional reagents available, including those that are photoactivated

The use of MALDI/MS for the analysis of multi-subunit complexes will increase in future years as practitioners learn more about how to prepare samples under non-denaturing conditions and more about the ionization mechanisms of the technique. The basic instrumentation is capable of performing the task quickly, reliably and accurately, allowing the investigator to deal with the major problem, the chemistry and structure of the protein itself.

REFERENCES

1. R. R. O Loo, D. R. Goodlett, R. D. Smith and J. A. Loo., *J. Am. Chem. Soc.* **115**, 4391 (1993).
2. J. A. Loo, *Mass Spectrom. Rev.* **16**, 1 (1977).
3. E. C. Huang, B. N. Pramanik, A. Tsarbopoulos, P. Reichert, A. K. Gauguly, P. P. Trotta and T. Nagabhushan, *J. Am. Soc. Mass Spectrom.* **4**, 624 (1993).
4. A. K. Gauguly, B. N. Pramanik, A. Tsarbopoulos, T. R. Covey and E. Huang, *J. Am. Chem. Soc.* **114**, 6559 (1992).
5. C. V. Robinson, E. W. Chung, B. B. Kragelund, J. Knudsen, R. T. Aplin, F. M. Poulsen and C. M. Dobson, *J. Am. Chem. Soc.* **118**, 6643 (1996).
6. L. Stryer, *Biochemistry*, 4th edn. Freeman, San Francisco (1995).
7. C. K. Mathews and K. E. van Holde, *Biochemistry*, Benjamin/Cummings, Redwood City, CA (1990).
8. T. E. Creighton, *Proteins, Structure and Molecular Properties*. Freeman, San Francisco (1984).
9. D. E. Schumm, *Essentials of Biochemistry*. F. A. Davis, Philadelphia, PA (1988).
10. A. Fersht, *Enzyme Structure and Mechanism*, 2nd edn. Freeman, San Francisco (1985).
11. M. Karas, U. Bahr and F. Hillenkamp, *Int. J. Mass Spectrom. Ion Process* **78**, 53 (1987).
12. M. Karas, U. Bahr, A. Ingendoh, E. Nordhoff, B. Stahl, K. Strupat and F. Hillenkamp, *Anal. Chim. Acta* **241**, 175 (1990).
13. M. Karas and U. Bahr, *Trends Anal. Chem.* **9**, 321 (1990).
14. F. Hillenkamp and M. Karas, in *Proceedings of the 37th ASMS Conference on Mass Spectrometry and Allied Topics*, Miami, FL, 1989, pp. 1168–1169.
15. M. Moniatte, F. G. van der Goot, J. T. Buckley, F. Pattus and A. van Dorsselaer, *FEBS Lett.* **384**, 269 (1996).
16. B. Rosinke, K. Strupat, F. Hillenkamp, J. Rosenbusch, N. Dencher, U. Kruger and H.-J. Galla, *J. Mass Spectrom.* **30**, 1462 (1995).
17. L. R. H. Cohen, K. Strupat and F. Hillenkamp, *J. Am. Soc. Mass Spectrom.* **8**, 1046 (1997).
18. T. B. Farmer, PhD Thesis University of Texas–Houston Health Science Center (1996).
19. A. S. Woods, J. C. Buchsbaum, T. A. Worrall, J. M. Berg and R. J. Cotter, *Anal. Chem.* **67**, 4462 (1995).
20. B. N. Pramanik, E. Huang, A. Tsarbopoulos, B. M. Girijavabhan, W. Windsor, T. B. Farmer, R. M. Caprioli and A. Ganguly in *Proceedings of the 44th ASMS Conference on Mass Spectrometry and Allied Topics*, Portland, OR, 1996, p. 986.
21. M. O. Glocker, S. H. J. Bauer, J. Kast, J. Volz and M. Przybylski, *J. Mass Spectrom.*, **31**, 1221 (1996).
22. G. E. Davies and G. R. Stark, *Arch. Biochem. Biophys.* **149**, 1 (1970).
23. T. B. Farmer and R. M. Caprioli, *Biol. Mass Spectrom.* **20**, 796 (1991).
24. B. Pramanik, unpublished results.
25. D. L. Smith, Y. Deng and Z. Zhang, *J. Mass. Spectrom.* **32**, 135 (1997).