Analysis of Protein–Protein Interaction Surfaces Using a Combination of Efficient Lysine Acetylation and nanoLC-MALDI-MS/MS Applied to the E9:Im9 Bacteriotoxin—Immunity Protein Complex

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To understand how proteins perform their function, knowledge about their structure and dynamics is essential. Here we use a combination of an efficient chemical lysine acetylation reaction and nanoLC-MALDI tandem mass spectrometry to probe the accessibility of every lysine residue in a protein complex. To demonstrate the applicability of this approach, we studied the interaction between the DNase domain of Colicin E9 (E9) and its immunity protein Im9. Free E9 and E9 in complex with Im9 were rapidly acetylated, followed by proteolytic digestion and analysis by LC-MALDI-TOF/TOF MS/MS. Acetylated peptides could be filtered out of the complex peptide mixtures using selective ion chromatograms of the specific immonium marker ions. Additionally, isobaric acetylated peptides, acetylated at different sites, could be separated by their LC retention times. The combination of LC and MALDI-TOF/TOF MS/MS provided information about the amount of acetylation on each individual lysine even for peptides containing several lysine residues. In general, our data agree well with those derived from the crystal structure of E9 and the E9:Im9 complex. Interestingly, next to in the binding interface expected lysines, K89 and K97, two from the crystal structure data unexpected lysines, K81 and K76, were observed to become less exposed upon Im9 binding. Moreover, K55 and K63, positioned in the predicted DNA binding region, were also found to be less accessible upon Im9 binding. These findings may illustrate some of the described differences in the solution-phase structure of the E9:Im9 complex compared with the crystal structure. (J Am Soc Mass Spectrom 2006, 17, 983–994) © 2006 American Society for Mass Spectrometry

Hydrogen-Deuterium exchange (HDx) in combination with mass spectrometry is one of the most frequently used methods to probe protein structure, conformational dynamics and protein–protein interactions [1]. HDx studies are primarily focused on the exchange of backbone amide hydrogens, enabling the dynamics of exchange to be monitored for the entire protein. The nonspecificity of D₂O helps to achieve a high coverage and spatial resolution; potentially, the exchange dynamics of each individual amino acid in the sequence can be monitored. A drawback of the HDx method is the possible fast back-exchange of deuterium to hydrogen, which restricts the use of HDx to short sample preparation times at low-temperature

and pH conditions. To monitor HDx, usually pepsin digestion at pH 2.8 is used. Since pepsin is a nonspecific protease, it leads to a large, unpredictable number of peptide possibilities, complicating data analysis [2, 3]. Additionally, when using tandem mass spectrometry on the (partly) H/D-exchanged proteolytic peptides, gas-phase “hydrogen-deuterium scrambling” may hamper the detailed analysis of amino acid specific exchange rates [4–7].

To overcome some of these restrictions, other chemical labeling methods have been developed. Many of them utilize the reactive primary amine on the side chain of lysine residues [8–10]. Since lysine residues are charged, they are most likely to be present at solvent accessible regions of proteins, making them ideal candidates to probe protein–protein interaction surfaces. For instance, Przybylski et al. described a method to probe protein structure by acetylation of lysines using acetic anhydride [8]. The acetic anhydride targets quite specifically the acetyl group of lysines, and covalently

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attaches an acetyl group in a nonreversible manner. Side reactions on the hydroxyl group of tyrosines have been reported, but they may be easily reversed under acidic conditions. Others have also successfully used acetic anhydride for structure determinations [9, 10]. Recently, several new reagents and labels have emerged that target solvent accessible lysines: N-hydroxysuccinimido-biotin (NHS-biotin) [11, 12], sulfosuccinimidyl acetate [13] and S-methylthioacetimidate [14]. Especially, the use of NHS-ester chemistry in lysine labeling has proven to be efficient and reliable. Compared to acetic anhydride, the used concentrations are 1000-fold lower to achieve ample lysine acetylation.

In this work, we chose for N-acetyl-succinimide (NHS-Ac) as a fast and efficient chemical probe to study the protein–protein interaction surface of the DNase domain of Colicin E9 (E9) with its immunity protein Im9. E9 is a member of a family of bacterial endonuclease colicins that are released by *Escherichia coli* into the extracellular medium during times of nutrient or environmental stress [15]. The highly toxic 15-kDa C-terminal DNase domain is translocated into the cytoplasm of the target cell in which it can accomplish its lethal effect by the hydrolysis of chromosomal DNA [16]. Under normal conditions, colicin-producing bacteria protect themselves from suicide by co-expressing a cognate immunity protein [17]. The toxic 15-kDa C-terminal DNase domain of E9 forms a tight hetero-dimeric complex with its cognate 9.5 kDa immunity protein Im9 with a dissociation constant of $\sim 10^{-16}$ M [15, 18]. The molecular determinants of this strong interaction have been studied in-depth by X-ray crystallography and comparative alanine scanning [19]. The DNase domain can be isolated as an active enzyme [20, 21] and its structure has been analyzed by X-ray crystallography in presence and absence of its cognate immunity protein Im9 [19]. The solution phase structure of free Im9, and bound to E9, has also been studied by NMR [22]. The 15-kDa C-terminal DNase domain of E9 is able to coordinate several divalent metal ions within its active site [20], although it is less clear what the role of the metal ions is in the DNase activity. It is, however, known that metal ion binding induces conformational changes in the E9 protein and stabilizes its structure [23].

To determine the surface accessibility of a free protein or one involved in a protein–protein complex, after a surface labeling method the protein is generally proteolytically cleaved and the resulting peptides analyzed by mass spectrometry. For complex peptide mixtures, the incorporation of a further liquid chromatography (LC) separation step before MS analysis may be essential. In recent years commercial LC-MALDI interfaces have become available. LC-MALDI can be used to obtain increased proteome coverage by applying the complementary combination of LC-ESI-MS/MS and LC-MALDI-MS/MS [24, 25]. Related to this work, Li et al. used LC-MALDI-MS/MS in combination with isotope labeling and limited enzymatic digestion to mon-

itor conformational changes of cardiac troponin C induced by calcium binding [26].

Here we present a combination of chemical lysine acetylation and LC-MALDI-MS/MS, which we believe to have intrinsic advantages over LC-ESI-MS/MS. This is achieved by utilizing the intense specific immonium ions of acetylated lysine residues (m/z 126.1 Da) under high-energy collisional activation conditions as present in MALDI TOF-TOF tandem mass spectrometry [27–29]. Our study reveals that extracted ion chromatograms (EIC) of the marker immonium ions are efficient tools to filter out acetylated peptides from the very complex peptide mixtures. We used N-acetyl-succinimide (NHS-Ac) as an efficient chemical probe to study the protein–protein interaction surface of E9 with its immunity protein Im9. By using the data filtering method based on the 126.1 Da immonium ions, and LC MALDI TOF-TOF on all observed acetylated peptides, we were able to probe each individual lysine residue in the protein complex.

Experimental

Materials

All chemicals were purchased from commercial sources and were of analysis grade, unless stated otherwise. NHS-Ac was synthesized by DCC/DMAP coupling of acetic acid to N-hydroxysuccinimide and stored under dry conditions in a dessiccator at room-temperature (RT). E9 and Im9 were expressed in *Escherichia coli* and purified according to Pommer et al. [20]. Endoproteinase Glu-C (V8) and chymotrypsin were purchased from Roche (Mannheim, Germany). Trifluoroacetic acid (TFA) was obtained in 1 mL vials from Sigma-Aldrich (St. Louis, MO), α -cyano-4-hydroxycinnamic (CHCA) acid and sinapinic acid were purchased from Fluka, (Buchs, Germany). HPLC-S gradient grade acetonitrile (AcN) was purchased from Biosolve (Valkenswaard, The Netherlands) and high purity water obtained from a Milli-Q System (Millipore, Bedford, MA) was used for all experiments. ZipTip μ -C18 tips were purchased from Millipore.

Acetylation of E9 and E9:Im9

Lyophilized E9 and Im9 were dissolved separately in water, and their concentration was assayed by absorption at 280 nm. A 2 μ M solution of E9 was prepared in 190 μ L buffer A (100 mM Na_2CO_3 , pH 8.0) in presence or absence of 2 μ M Im9 and in presence or absence of 100 μ M ZnSO_4 . NHS-Ac was dissolved in AcN to a concentration of 2 M and subsequently diluted 10-fold in buffer A. Ten μ L of the diluted reagent was added to the E9 solution and reacted at RT for 3 min (see also Figure 1). The reaction was allowed to quench for 20 min by addition of 200 μ L buffer B (100 mM $(\text{NH}_4)_2\text{CO}_3$, pH 8.0). To ease in solution digestion of E9, the reaction

mixture was heat inactivated at 65 °C after addition of 100 μ L buffer C (100 mM Na_2CO_3 , 25 mM EDTA, pH 8.0).

Sample Preparation

For MALDI-TOF-MS analysis on intact proteins, 10 μ L reaction mixture was acidified by addition of 1% TFA and applied to a ZipTip. Desalted samples were directly eluted on the MALDI target with 0.8 μ L matrix solution A (5 g L^{-1} sinapinic acid in 80% AcN, 0.1% TFA). For peptide mass fingerprinting, samples were digested overnight with a 1:50 protease to E9 mass ratio. V8 digests were performed at RT, chymotrypsin proteolysis was performed at 37 °C. After digestion samples were stored at –30 °C until analysis. For analysis, 5 μ L of acidified peptide mixture was trapped on a ZipTip, desalted and eluted in 0.8 μ L matrix solution B (5 g L^{-1} CHCA in 80% AcN, 0.1% TFA) onto the MALDI target.

Instrumental

MALDI-TOF-MS analysis on intact proteins was performed on an Applied Biosystems 4700 Proteomics analyzer (Framingham, MA) in linear mode, using a m/z 5000–25,000 mass range with a focus m/z of 7500. MALDI-TOF-MS of peptide mixtures was performed on the same machine in reflectron mode using a m/z 700–5000 Da mass range. Data were acquired at a 200 Hz laser repetition rate, a 20 kV accelerating voltage, a 70% grid voltage and a digitizer bin size of 1.0 ns.

The nanoLC system consisted of a Famos autosampler, an Ultimate nano HPLC system, and a Rheodyne automated switching nanovalue all from LC Packings (Amsterdam, The Netherlands). The nanoLC system was controlled by Chromeleon 6.60 software (Dionex, Bavel, The Netherlands). A blank MALDI target plate (Applied Biosystems) was spotted with the LC eluent using a Probot micro fraction collector (LC Packings) controlled by μ Carrier V 1.68 software (Dionex). In the nanoLC-system two homemade columns were placed: a 100 μm i.d./375 μm o.d. precolumn of 19 mm filled with 5 μm , 200 Å Aqua C_{18} stationary phase (Phenomenex, Torrance, CA) and a 75 μm i.d./360 μm o.d. analytical column of 150 mm filled with the same material both packed using the procedure described by Pinkse et al. [30]. In this set-up, connections were made using two PEEK zero dead-volume T-pieces (Inacom,

Figure 1. Schematic overview of the experiment. E9 and E9:Im9 complex were subjected to selective acetylation with NHS-Ac. After quenching, the proteins were digested by chymotrypsin or V8 protease to reach high sequence coverage. Next, the peptides were separated by nanoLC and spotted onto a MALDI plate. All spots on the MALDI plate were then analyzed by MALDI-TOF/TOF. Extra selectivity and sensitivity for peptides that contained acetylated lysines could be obtained by filtering the data for the MS/MS product with m/z 126.1, a specific immonium ion of acetylated lysine.

Veenendaal, The Netherlands), one Teflon sleeve (LC Packings), and fused silica capillary (75 μm i.d./375 μm o.d., Bester, Amstelveen, The Netherlands) except for the connection between the column and the Probot for which a fused silica capillary with 20 μm i.d./280 μm o.d. was used (LC Packings). The fused silica needle of the Probot had an i.d. of 30 μm (LC Packings). MALDI-MS(/MS) was performed using a proteomics analyzer controlled by the 4700 Explorer 3.0 software (Applied Biosystems).

NanoLC-probot

NanoLC was conducted using the set-up similar to that described by Meiring et al. [31]. For nanoLC-MALDI, the set-up depicted in Figure 1 and described in the following section was used. Before injection, 10 μL of the sample containing the digest of E9, acetylated E9 or the acetylated E9:Im9 complex was acidified with 10 μL 1% TFA to allow for optimal trapping. The 20- μL sample was injected onto the pre-column using Solvent A (5% AcN, 95% water, and 0.05% TFA) at 5 $\mu\text{L min}^{-1}$. The valve was positioned so that the restriction capillary, connected to the first T-piece, was blocked and all LC eluent was flushed through the pre-column. The capillary on the second T-piece was connected to waste. As a result, no eluent was flushed through the analytical column during this step. After trapping the sample, the pre-column was flushed for 10 min with Solvent A at the same flow. Next, the nano-valve was switched with the intention that the pre-column was positioned in-line with the analytical column. Subsequently, the flow was raised in 1 min to 200 $\mu\text{L min}^{-1}$. Because with this valve position the restriction capillary was connected to the waste line, a 1:1000 split was built into the system. Consequently, the flow over the combined pre-column and analytical column was 200 nL min^{-1} . After switching the nano-valve, the gradient was started using a binary solvent system: Solvent A as described above and Solvent B (95% AcN, 5% water, and 0.05% TFA). The gradient ran as follows: during the first 60 min Solvent B was increased from 0 to 55%, afterwards Solvent B was increased to 90% in 1 min and maintained at that percentage for another 5 min. Finally, the system was reconditioned by decreasing Solvent B to 0% in 1 min and 0% B was maintained for 25 min. Afterwards, the flow was returned in 1 min to 5 $\mu\text{L min}^{-1}$ and the nano-valve was switched to its original position. Accordingly, the pre-column was positioned off-line to the analytical column.

The Probot, a micro fraction collector, was started 15 min after injection. Using the Probot, the LC eluent, 200 nL min^{-1} , was mixed with 1.3 $\mu\text{L min}^{-1}$ of MALDI matrix (75% AcN, 25% water, 0.1% TFA, and 3 mg/mL CHCA) and spotted on a blank MALDI target. Sample was spotted, following a predefined 18×18 array, every 20 s during 90 min, resulting per LC run in 270

spots of 500 nL. The spotted MALDI target was measured off-line using MALDI-TOF/TOF.

MALDI-TOF/TOF

The 4700 proteomics analyzer (Applied Biosystems) was used for all MALDI-TOF/TOF measurements. The software was equipped with LC-MALDI features wherein all spots were regarded as one large sample spot set. Of each LC run, MS and MS/MS on the five most abundant precursors were conducted in positive ion mode. MS, used in reflectron mode, was used to scan a m/z -range of 900–5000 with a fixed laser intensity and 4500 laser shots per spectrum. Each spot was calibrated by using external calibration spotted in a separate sample spot. MS/MS was conducted using 1 kV collision energy and air as collision gas. MS/MS was measured until a S/N ratio of 30 was obtained for at least 20 peaks in the spectrum with an upper limit of 15,000 shots per spectrum. Data were analyzed using the 4700 Explorer software.

Data Analysis

Solvent accessibilities of E9 lysine residues were based on the X-ray data of E9 (accession code: 1FSJ) and E9:Im9 complexes (accession codes: 1EMV [19], 1BXI [32], and 1FR2 [19]) and calculated by using the software program GETAREA 1.1 (www.scsb.utmb.edu/getarea) [33]. We calculated both the total solvent accessibility of the lysine side chains and the reactive NZ atoms by using a radius of the water probe of 1.4 Å. The average solvent-accessible surface areas of a lysine side chain and a NZ atom in the “random coil” tripeptide Gly-Lys-Gly in an ensemble of 30 random conformations are 164.5 and 45.0 Å², respectively. Lysine side chains were considered to be solvent exposed when the area was larger than 82.25 Å². Subsequent percentages were calculated by dividing the observed area with the total area of 164.5 or 45.0 Å².

The MALDI-TOF spectra of proteins were analyzed using the Data Explorer software (Applied Biosystems). Data from the protein digests were analyzed with the same software. Briefly, obtained peak lists were mass fingerprinted by using an E9:Im9 protein database in combination with the Mascot search engine (Matrix Science, www.matrixscience.com). Peptides were matched within 25 ppm and with optional modifications of oxidation on methionine-residues and acetylation on lysines and the N-terminus of the protein. For LC-MALDI-data, the Peak Explorer software (Applied Biosystems) was used to obtain a base peak chromatogram (BPC) and extracted ion chromatograms (XIC) of precursor ions of the MS data. Quantification of precursor ions was conducted by using the area of peaks obtained from the XIC. Using the Analysis tab of the Spot set window, MS/MS data could be searched for the specific acetylated lysine immonium ion at m/z 126.1 Da, with a deviation of 0.1 Da to obtain an extracted ion

chromatogram of the MS/MS data, which will be abbreviated with EIC to distinguish with the extracted ion chromatogram from the MS data. The obtained LC-MS and LC-MS/MS data were further analyzed using the MASCOT search engine.

Two types of relative quantification were performed. First, normalization of the acetyl occupancy was obtained for all peptides. This was achieved by analyzing the peak areas of a certain peptide with all possible occupancies, i.e., with none, one, two, etc. acetylations. Then, the areas of all the different species of a peptide were put as the fraction of the summed areas, followed by normalization. An example of this calculation is given in the results and discussion section for Figure 3. Second, the percentage of acetylation for each lysine was determined using the combined data of the MS/MS spectra and the normalized quantification data. The MS/MS spectra were used to determine the exact position of acetylation, while the normalized quantification data showed the ratio of the same peptide with different numbers of acetylation. For example, when a peptide had two lysines, the MS/MS data of the 1-fold acetylated peptide provided information about the predominantly acetylated lysine. By combining this knowledge with the fraction of non-acetylated, 1-fold acetylated and 2-fold acetylated peptide, the average percentage of acetylation for each lysine could be determined individually. The standard deviation (Stdev) on this average percentage of acetylation for a lysine was calculated using data from duplo samples. When a specific lysine was retrieved in more than one peptides stretch, the average percentage of acetylation per lysine in the separate peptides was averaged and the standard deviation (Stdev) was determined.

Results and Discussion

NHS-Ac is an Efficient Acetylating Reagent

The method employed here is based on the availability of lysine residues for reaction with an acetylating reagent. As lysine residues have basic, charged side chains, they are mainly found on the surfaces of proteins, making them ideal targets to probe protein–protein interaction surfaces.

Our first aim was to investigate the acetylating properties of NHS-Ac by assessing whether lysine residues are affected by reaction with the reagent. To probe the interaction surface properly, full acetylation of all available lysine residues in E9 within a short period of time is required. Three different excess ratios of NHS-Ac (2.5, 25, and 250 mol/mol lysine residues) on E9 were tested. To elucidate which minimum reagent concentration resulted in complete modification of E9, MALDI-TOF-MS was employed. First, a spectrum was obtained of a 1:1 mixture of unmodified E9:Im9, which was used as a control measurement. The $[M + H]^+$ (15,088 Da) and $[M + 2H]^{2+}$ (7545 Da) signals of E9 were observed, as well as the $[M + H]^+$ (9583 Da) signal of Im9 at their corresponding theo-

retical average mass (Figure 2a). Upon modification with a 250-fold excess of NHS-Ac for 3 min at room temperature, the $[M + 2H]^{2+}$ -signals of E9 shifted up in m/z to give the distribution shown in Figure 2c. The most intense peak in the distribution was observed at 7986 Da. The others were all found at 21 Da m/z -intervals of this peak. This corresponds to an overall mass difference of 42 Da, the mass increase of an acetylated lysine. The average number of modifications was calculated from the observed mass as follows: (7986 Da–7545 Da)/21 Da = 21 modifications. This number corresponds closely to acetylation of every lysine and the N-terminus. The same shift could be observed for the $[M + H]^+$ -signal of E9, however, the resolution on this signal was not ideal to determine the exact modification numbers (data not shown). Modification of E9 with the two lower excesses did not lead to complete lysine acetylation within 3 min (data not shown). Therefore, for further experiments, a 250-fold excess of NHS-Ac and a reaction time of 3 min was used.

These results, obtained for the unbound E9, are in close agreement with the crystal structure [19]. The X-Ray structure revealed that all 20 lysines and the N-terminus were calculated to be solvent accessible (50–100%) (Table 1). The percentage values were calculated with the GETAREA software as described in the materials and methods. Three very similar crystallographic models of E9 in complex with Im9 (pdb-files: 1EMV, 1BXI, and 1FR2) were probed for solvent accessibility of their lysine side chains and their NZ atoms. The solvent accessibilities of the side chains were very similar in the three models, whereas the accessibilities of the NZ atoms were very different in each model (data not shown). These differences in the accessibilities of the NZ atoms are probably related to the dynamics of the residues on the surface of E9 DNase. Therefore, we used the values of the side chains as a measure for the solvent accessibilities (Table 1).

MALDI-TOF-MS Analysis Reveals a Clear Im9 Induced Protection

Subsequently, acetylation of E9 was assayed in the presence of Im9. Under identical conditions, the most intense peak of the $[M + 2H]^{2+}$ signals of E9 now shifted up to $m/z = 7838$ Da, corresponding to on average 14 lysine modifications, instead of 21 (Figure 2d). These data indicate that Im9 reduces the acetyl occupancy of several lysine residues in E9. Since Im9 also has six lysine residues, modification of Im9 alone (Figure 2f) was compared with acetylation of Im9 in the presence of E9 (Figure 2g). No significant effect of E9 on the overall acetyl occupancy of Im9 lysines was observed. This is in agreement with the NMR studies on Im9 alone and Im9 in the presence of E9. These indicated that binding E9 to Im9 does not cause a major conformational change in Im9 and that lysine residues of Im9 are not directly involved in the interaction with E9 [22]. Unfortunately, no NMR structure could be

Figure 2. E9:Im9 interaction reduces the acetyl occupancy of several E9 lysine residues while Im9's lysine residues are not affected. (a) MALDI TOF MS spectrum of a 1:1 mixture of E9 and Im9. Signals shifted to higher m/z upon acetylation with NHS-Ac. (b) Zoomed in spectra of the $E9^{2+}$ ion signal, shifted differentially in absence (c) and presence (d) of Im9. No differential shift was observed for the $Im9^{1+}$ peak (e) in absence (f) or presence (g) of E9.

resolved for E9, as a consequence of the high spectral complexity observed for E9 [34, 35].

The effect of transition-metal ions on the activity and stability of E9 and the E9:Im9 complex have been studied in detail [20, 23, 36]. In our experiments, we did not observe any significant differential effect on the extent of acetylation induced by the presence of Zn^{2+} ions, both in absence and presence of Im9 (data not shown). This is in line with the fact that the crystal structures of the E9-Im9 complex in presence and absence of Ni^{2+} ions are very

similar. Kühlmann et al. state that the structural rearrangements that resulted from metal ion binding are minimal and only affect the structure in the immediate vicinity of the metal ion binding site [19].

A MALDI-TOF Fingerprint of Digested E9:Im9 Complex Shows Better Resolution

To investigate the effect of Im9 binding to E9 in more detail, the protein samples were digested with V8

Figure 3. Peptide mass fingerprinting reveals regions within E9 with reduced acetyl occupancy. MALDI-TOF MS spectra of the E9 chymotryptic peptide SPTFPKNQQVGGRKVY (84-99). (a) Untreated, acetylated E9 in absence (b) and presence (c) of Im9.

and/or chymotrypsin. Although trypsin is the most reliable and robust protease, its use was omitted here as it can no longer cut after an acetylated lysine. Using trypsin (data not shown), the proteolytic peptides became very large, thereby reducing our resolution and detection efficiency.

In the V8-digest of E9, 12 lysine-containing peptides were identified by mass-fingerprinting, covering 11 lysines and 66% of the total protein sequence. The same method yielded nine peptides for the chymotrypsin digest, covering 17 lysines and the N-terminal amine group with a sequence coverage of 78% (data not shown). Combining the V8 and chymotrypsin data, 17 out of 20 lysines of E9 and the N-terminus were covered, with 82% total sequence coverage. The amino acid stretch between L23 and E46, containing K28, K41, and K45, was not covered.

By comparing the E9 derived peptides in the presence and absence of Im9, more detailed information about the area of E9 involved in the interaction became evident. This is illustrated in Figure 3: a typical example of a chymotryptic E9-peptide (84-99, 1805.97 Da) with reduced acetylation induced by the binding to Im9. In absence of Im9, the mass of the peptide was shifted 84 Da to 1890.0 Da, corresponding to complete acetylation on both lysines. The lysines present in this peptide became less accessible for acetylation upon Im9-binding, as the same peptide was now found in three forms; unacetylated, singly acetylated, and doubly acetylated. A semiquantitative analysis was performed by taking the areas under the peak of the individual ions normalized to the total area of all three ion peaks. This resulted in 43% intensity allocated to unacetylated, 51% intensity to singly acetylated, and only 6% to doubly acetylated. Now the acetyl occupancy, or average modification percentage, of this peptide was calculated at 32% $[(0 \cdot 0.43 + 1 \cdot 0.51 + 2 \cdot 0.06)/2]$ in the presence of Im9 and 100% in the absence. This means a 68% protection for this peptide towards the acetylating reagent upon Im9 binding. A similar semiquantitative analysis was conducted for all other observed peptides. If a lysine was

covered in multiple peptides, for instance resulting from the two different digests, the contribution was averaged. It is clear from the spectra shown in Figure 3 that K89 and K97 are involved in the interaction of E9 and Im9. The V8 peptide 67-100 confirmed this as its acetyl occupancy dropped from 100 to 44% in the presence of Im9. In most detected peptides there were two or more lysine residues present, hampering an in-detail analysis by just MALDI-TOF-MS fingerprinting. Such analysis alone does not enable to assess what the individual contributions of the two isobaric species present in Figure 3, acetylated at K89 or at K97 alone, to the peak area at m/z 1847.98 Da are.

To address this issue, we chose for a LC-MALDI-TOF/TOF approach, which allowed us to separate isobaric species by their differential elution properties. Subsequent sequencing by MS/MS can specifically identify the structure of the isobaric species. Using this approach, we observed that it was possible to estimate the separate contributions of each isobaric species, providing a high-resolution picture of, by the presence of Im9 affected, lysine residues in E9. Also, all six lysine residues of Im9 were covered using the two different digests. However, no differentially modified peptides were observed, reiterating that the acetyl occupancy of Im9's lysine residues was not affected by binding to E9 (data not shown). As mentioned earlier, this was as expected, based on the available X-ray [19] and NMR structures of Im9 [22] in absence and presence of E9.

Data Filtering Using Immonium Marker Ions Efficiently Identifies Acetylated Peptides

By including an additional separation technique such as nanoLC before MS, we aimed to separate isobaric acetylated peptides, like the ones shown in Figure 3. Adding the LC-approach to our analysis increased the sequence coverage of E9 to 95%, likely due to less ion suppression, and an important additional peptide containing K41 and K45 could be detected. Using nanoLC-

Table 1. Acetyl occupancy of each lysine, as calculated from the nanoLC-MALDI-TOF/TOF data as described in The Materials and Methods section (left) and solvent accessibility of the lysine side chains as calculated from the X-ray data (right). E9 lysine residues that showed major changes in occupancy or solvent accessibility upon Im9 binding are presented in bold

Lysine	Acetyl Occupancy by MS (%)				Solvent Accessibility by X-Ray (%)	
	E9		E9:Im9		E9	E9:Im9
	Avg	Stdev	Avg	Stdev	(1FSJ.pdb)	(1EMV.pdb)
NT	97.0	1.2	86.0	2.4	99.7	^b
K4	97.0	1.2	86.0	2.4	67.0	61.2
K7	97.0	1.2	86.0	2.4	75.0	84.1
K10	97.0	1.2	86.0	2.4	72.5	65.5
K14	97.0	1.2	86.0	2.4	93.7	74.8
K16	97.0	1.2	86.0	2.4	65.8	66.6
K21	97.0	1.2	86.0	2.4	75.9	80.7
K28	^a		^a		68.2	64.4
K41	11.6	1.4	13.8	10.3	58.6	48.6
K45	87.1	18.3	100.0	0.0	57.2	56.5
K48	97.8	1.8	90.1	6.2	90.8	88.9
K55	88.9	12.7	47.3	5.6	56.9	61.4
K63	100.0	0.0	47.2	4.0	67.6	70.8
K69	100.0	0.0	90.7	2.8	46.5	80.2
K76	100.0	0.0	10.5	3.5	50.5	45.7
K81	83.4	23.6	0.1	0.1	61.2	50.5
K89	100.0	0.0	54.4	10.4	100.0	50.2
K97	100.0	0.0	0.8	0.5	55.3	9.5
K105	96.4	5.4	90.5	13.3	66.8	62.4
K125	96.0	8.0	94.1	3.4	55.5	57.2
K134	94.7	9.1	95.5	2.4	100.0	^b

^aNot covered in digests.

^bResidue not present in crystallographic model.

MALDI-TOF/TOF, the obtained tandem MS data could be filtered for specific detection of acetylated peptides by using the specific immonium marker ion of an acetylated lysine that is formed via collision induced dissociation at m/z 126.1 Da (CID) [27–29]. This ion is a further fragment ion induced by the loss of NH_3 from the acetylated lysine immonium ions at m/z 143.1. However, the 126.1 Da marker ion is thought to be more specific and ~ 10 times more sensitive than the immonium ion at m/z 143.1 [28]. Some of the advantages of using this filtering procedure become evident when the normalized base peak chromatograms (BPC) are compared with the normalized extracted ion chromatogram (EIC) of the 126.1 Da ions (Figure 4, upper two chromatograms). Overlaps of the BPC and the EIC could be found for some peaks, e.g., spots 57, 70, and 76. However, more careful examination of the EIC between spot 42 and 54 showed that two acetylated precursor ions were present in spots 44 and 46 that were obscured under a larger coeluting peak in the BPC. Also, the intense BPC peak in, e.g., spots 48 and 62, could be discarded as acetylated peaks as they did not show any signal in the 126.1 Da EIC. These examples show that creating an EIC for acetylated lysines by its specific marker immonium ion was an advantageous approach to quickly filter out peptides of interest. The filtering procedure increased both selectivity and sensitivity for the determination of acetylated peptides. This is further illustrated in Figure 4 (lower two chromatograms)

Figure 4. Efficient selected filtering based on the acetylated lysine marker ion. The LC-MALDI BPC chromatogram of the V8 digest of the acetylated E9:Im9 complex is shown in the top trace. Below the EIC of the acetylation marker ion with m/z 126.1 Da is shown, which directly identifies acetylated peptides present in the complex mixture. The third trace shows the selected ion chromatogram of the precursor ion at m/z 1719.9 Da, which shows three ions with different retention times. The bottom trace shows that with further filtering by using both the 126.1 marker ion and the ion chromatogram of the precursor ion at m/z 1719.9 Da, it can be exclusively verified that only the precursor in spot 70 contained an acetylated lysine.

Figure 5. LC MALDI TOF/TOF enables the elucidation of the exact structure of two isobaric peptides to pinpoint the locations of lysine acetylation. (a) Extracted ion chromatogram of ions with m/z 1845.8 Da, i.e., KSFDDFRKAVWEE (47–60) with one acetyl group (dashed line). (b) MS/MS spectra corresponding to precursor ion 1845.8 Da in LC-MALDI spot 70 and (c) in LC-MALDI spot 72. The spot 70 spectrum was identified to originate from the FKSFD-DFRK(Ac)AVWEE ion, while the spot 72 spectrum corresponded to apparently 10-fold more abundant FK(Ac)SFDDFRKAVWEE ion. Relative abundance was estimated based on the ion intensity in the EIC of the y_8 [(a), thick line] and y_8 +Ac [(a), thin line] ions in the two MS/MS spectra.

when zooming in on the peptide precursor ion with a m/z of 1719.9 ± 0.3 Da, corresponding to the rather intense peak at spot 70 in the BPC. In the XIC of this precursor ion, three peptide ion peaks could be observed in the chromatogram (Figure 4). When the XIC data were combined with the EIC data of 126.1 Da, one peak remained, showing the increased selectivity and sensitivity.

In a further analysis of the data, the acetylated peptides identified by the filtering method were matched to their original precursor mass. For each identified peptide, an XIC for their precursor mass was created and the corresponding MS/MS spectra were then analyzed. From the XIC it is now possible to assess different isobaric species by interpreting the MS/MS spectra in each individual XIC peak. With this approach, it is possible to precisely localize the position of the acetylated lysine within each peptide. An example of this approach is depicted in Figure 5 for the precursor ion with m/z 1845.8. This is the V8 peptide, FKSFD-DFRKAVWEE (47–60), with one acetyl group attached. The peptide contains two lysine residues and as a consequence two peptides could have been formed, whereby either K48 or K55 is acetylated. The XIC showed that the species with m/z 1845.8 eluted in two different LC-MALDI spots, 70 and 72 (Figure 5a, dashed line). Next, the MS/MS spectra of the 1845.8 Da precursor peptide ions present in spots 70 and 72 were compared (see Figure 5b and c, respectively). Both spectra contain the 126.1 Da immonium ion, but also show clear differences. The intense y_8 - and y_9 -ions are ideal for distinguishing these two isobaric species. In the MS/MS spectrum of precursor 1845.8 in spot 70, depicted in Figure 5b, the y_8 ion was observed at an m/z 1106.6, while the y_8 ion in the spectrum of spot 72 was

observed at m/z 1064.4, i.e., 42 Da lower (corresponding to the mass of one acetyl group). This indicates that the y_8 ion of the peptide eluting in spot 70 contains an acetyl group, while the one eluting in spot 72 does not. This, and other fragment ions observed in the spectra, define that the peptide in spot 70 originates from the peptide acetylated at K55, while Figure 5c depicts the spectrum of the peptide acetylated at K48. An EIC of the two different y_8 ions is depicted in Figure 5a as well. When assuming that both peptides have similar ionization efficiencies, it can be concluded from the EIC of 1064.4 Da (thick line) and 1106.4 Da (thin line) that the K48-acetylated peptide has a 10-fold higher intensity than the peptide with an acetyl on K55. This implies that K48 has a high acetyl occupancy compared to K55, which seems to be well protected upon Im9-binding.

The 67–100 Stretch Is Involved in the Interaction with Im9

The MALDI-TOF mass fingerprint indicated that the V8 peptide 67–100 (LSKNLNPSNKS SVSKGYSPFTPKN-QQVGGRK VYE) harbors interesting information about the interaction between E9 and Im9. This peptide was recovered in its completely acetylated form when Im9 was not present (data not shown). In contrast, in the presence of Im9, this peptide contained in between zero and four acetyl groups. Tandem mass spectra of all four acetylated species of this peptide were considered. To our surprise, we observed a strict order of acetylation for the five different lysines present in this peptide, i.e., no significantly abundant isobaric species could be observed for this peptide. From the MS/MS-spectrum of the singly acetylated peptide, we determined that

Figure 6. Consecutive acetylation events on E9-peptide 67-100 in presence of Im9. (a) MALDI TOF/TOF MS/MS spectrum of the 2-fold acetylated peptide, LSK(Ac)NLNPSNKSSSVSKGYSPFTPKNQQVGGRKVE (MW = 3822.7) (b) MALDI TOF/TOF MS/MS spectrum of the 3-fold acetylated peptide LSK(Ac)NLNPSNK(Ac)SSSVSKGYSPFTPKNQQVGGRKVE (MW = 3864.8). Identifying the exact location of the acetylation in these peptides enables the elucidation of consecutive acetylation events and, thus, the order of solvent protection of the individual sites upon E9 binding.

K69 was the uniquely acetylated residue (data not shown). K89 was found to be the next residue to be acetylated in the doubly acetylated peptide (Figure 6a). The subsequent lysine found to be acetylated was K76 (Figure 6b). The peptide with four acetyl groups was modified at K69, K76, K89, and K97 (data not shown). The fifth and last lysine, K81, was never found to be acetylated in the presence of Im9. Combining the normalized quantification data and the information obtained from the MS/MS spectra, the percentage of acetylation of each lysine could now be determined (Table 2). An overview of the nanoLC-MALDI-TOF/TOF derived acetyl occupancy of each individual lysine residue is given in Table 1. All lysines between 41 and 134 could be individually scored for their acetyl occupancy. The intensity of chymotryptic N-terminal peptide 1-22 was too low to obtain reliable MS/MS data and therefore the individual lysines could not be scored for this N-terminal stretch. However, this region does not seem to be affected by Im9 binding. Also depicted in Table 1 are the solvent accessibilities of each lysine residue side chain, as derived from the X-ray data of E9 and E9 bound to Im9 (accession codes 1FSJ and 1EMV [19], respectively) by using the GETAREA software.

LC-MALDI TOF/TOF Data Provides New Insight on the E9:Im9 Interaction

The derived experimental data on the acetyl occupancy of lysine residues in E9 were compared with the solvent accessibilities obtained from the X-ray structural models of E9 and the E9:Im9 complex (Table 1, Figure 7). The lysine occupancy and solvent accessibility percentages were sorted in five distinct categories between 0 and 100% and color coded into the model (Figure 7).

Our MS data show that lysine residues K55, K63, K76, K81, K89, and K97 become considerably more protected against acetylation upon interaction with Im9. The X-ray model of the structure of the E9:Im9 complex shows that the interaction surface between E9 and Im9 spans primarily the amino acids 72–98 of E9. In fact, the two lysine residues K89 and K97 are directly involved

Table 2. Percentage of acetyl occupancy on each individual lysine residue in the partial E9 sequence 67-100 (SKNLNPSNKSSSVSKGYSPFTPKNQQVGGRKVE) in the presence of Im9, as calculated from the nanoLC-MALDI-TOF/TOF data. See data analysis in the experimental section for calculation procedures

A) Quantification is given in column 3 and in column 4 the acetylated lysines are indicated			
Species	Number of Ac-groups	Acetyl occupancy (%)	Acetylated lysine
I	0	0.73	-
II	1	31.0	K69
III	2	48.8	K69, K89
IV	3	11.7	K69, K89, K76
V	4	1.14	K69, K89, K76, K97
VI	5	0	K69, K89, K76, K97, K81

B) Percentage of acetylation for each lysine		
Lysine	Present in:	Percentage of acetylation
K69	II-V	92.7
K89	III-V	61.7
K76	IV-V	12.9
K97	V	1.14
K81	-	0

Figure 7. Graphical representation to compare acetyl occupancy MS-data with X-Ray solvent accessibility data. Solvent accessibility data as extracted from the X-ray data of E9 and E9:Im9 using the program GetArea are given on the left (also see Table 1). On the right, the experimental data as obtained in this study on the lysine solvent accessibilities are given (see also Table 1). The amount of acetyl occupancy/solvent accessibility is given using a color scheme, whereby green indicates 0–20%, beige 20–40%, yellow 40–60%, orange 60–80%, and red 80–100% occupancy/accessibility.

in the interaction with Im9 [19]. The side chain of K89 forms a hydrogen bond interaction with the side chain of E41 of Im9, whereas the side chain of K97 coordinates towards the side chain of D51 of Im9. These interactions protect the two residues from solvent (Table 1). Our MS data on K89 and K97 are fully in line with these data, as they show a significant decrease in acetyl occupancy of K89 and K97 upon binding to Im9. The lysines K81 and K76 are also in the peptide stretch 72–98, but are, according to the X-ray models, not directly involved in the interaction with Im9. In addition, the model suggest that binding to Im9 does not influence the solvent accessibility of these two residues. However, our MS data clearly indicate that Im9 interaction lowers the acetyl occupancy of the two lysines significantly from fully reacted in the free E9 to nearly fully protected from our reagent in the Im9 bound state (Table 1). K76 and K81 are positioned in an α -helical domain in close proximity to Im9; therefore, we speculate that binding to Im9 induces changes in the dynamics of K76 and K81 such that they become protected for the reagent. Alternatively, it may also indicate that E9 in the E9:Im9 complex is partly present in a conformation different

from the one described in the X-ray model. This is not unexpected as solution-phase NMR data have indicated high structural dynamics throughout the whole structure of E9 in both free and Im9-bound state [34, 35]. Unfortunately, the complete NMR structure of E9 could not be solved due to these high structural dynamics.

We also observed that K55 and K63, which are both present in an α -helix, show a decreased lysine occupancy upon binding to Im9 (Table 1). These changes in accessibility were also not predicted from the X-ray structures. The rationale for the decreased accessibility is not clear, as both residues do not form a direct interaction with Im9. However, it is interesting to note that K55 is known to form a hydrogen bond with the dsDNA substrate in the structural homologue E7 [37]. Upon binding of Im9 to E9, the interaction with dsDNA is inhibited. The principle behind this inhibition is not well understood; however, it can be speculated that the interaction with Im9 also induces conformational dynamics in some amino acid residues, such as in K55 and K63, to hamper these residues to interact with dsDNA.

A final intriguing result is the low acetyl occupancy of K41 both in absence and presence of Im9. As this residue is far from the Im9 interaction surface and the catalytic center, we cannot give a rationale for the low occupancy of this residue. The low accessibility of this residue is in disagreement with the X-ray model (see Table 1), reinstating that the solution-phase structure of E9 in the presence and absence of Im9, might to some extent be different.

Conclusions

The combination of lysine acetylation, proteolytic digestion, and MALDI-TOF/TOF tandem mass spectrometry can be successfully applied to study protein–protein interaction surfaces. When nanoLC is used in combination with MALDI-TOF/TOF, data resolution can be increased and single amino acid resolution may be obtained. Extra selectivity and sensitivity for peptides that contain acetylated lysines can be obtained by filtering the data for the MS/MS product with m/z 126.1, a specific immonium ion of acetylated lysines. The applicability of this approach was demonstrated using the E9:Im9 complex. Our data clearly revealed that six lysine residues in the E9 protein become less accessible upon binding of Im9: K55, K63, K76, K81, K89, and K97. K76, K81, K89, and K97 are located at the protein–protein binding interface. The X-ray structure model predicts that K89 and K97 are directly involved in the binding with acidic amino acid residues of Im9. Our data suggested that K81 and K76 are involved in the E9:Im9 interaction, or at least in very close proximity to Im9. The other lysine residues with a reduced accessibility are K55 and K63, of which K55 is involved in dsDNA binding in the E7 homologue enzyme. In the E7:Im7 complex, Im7 binding physically blocks one of the major dsDNA binding sites [37]. Due to high homology among the colicins, the same situation is

expected for E9:Im9. It may also be hypothesized that the conformational dynamics of amino acids in the DNA interacting region change upon Im9 binding, thereby further hampering interaction with dsDNA.

In brief, the presented approach can be used to probe protein–protein interactions in moderate detail. This may be used as complementary data to already available structural models from X-ray crystallography. As it is a solution-phase approach, additional information may be obtained reflecting specific solution-phase conformations.

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