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Using LC-MS with de novo software to fully characterize the multiple methylations of lysine residues in a recombinant fragment of an outer membrane protein from a virulent strain of *Rickettsia prowazekii*

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Abstract

The outer membrane protein B (OmpB) of the typhus group rickettsiae is an immunodominant antigen and has been shown to provide protection against typhus in animal models. Consequently, OmpB is currently being considered as a potential rickettsiae vaccine candidate to be used in humans. The OmpB from virulent strains are heavily methylated while the attenuated strains are hypomethylated. Western blot analysis of partially digested OmpB revealed that one of the reactive fragments was located at the N-terminus (fragment A, aa 33–272). Recently, we have over expressed, purified, and chemically methylated the recombinant fragment A from *Rickettsia prowazekii* (Ap). The methylated Ap was thoroughly characterized by LC/MS/MS on the ProteomeX workstation. The protein sequence of Ap with and without methylation was 87.7% and 100% identified, respectively. This high sequence coverage enabled us to determine the sites and extent of methylation on the lysine residues in Ap. All the lysine residues except the C-terminus lysine were either mono-, di- or tri-methylated. In addition, carbamylation on the N-terminus glycine was identified using a combination of denovo sequencing (DeNovoX) and the pattern recognition (SALSA) program with accurate mass measurement. We demonstrated that the use of peptide identification (SEQUEST) in combination with SALSA and denovo sequencing provided a useful means to characterize the sequence and posttranslational modifications of given proteins.
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Keywords: *Rickettsia prowazekii*; Lysine methylation; SALSA; De novo sequencing; LC-MS; Ion trap

1. Introduction

Rickettsia prowazekii, the etiologic agent of epidemic typhus, has the potential to be selected as a biological weapon by terrorists and rogue countries because of its stability in dried louse feces and in its infection by inhalation of aerosol [1]. In 1975, the World Health Organization estimated 104,000 casualties, with 19,000 dead and 85,000 incapacitated, following a hypothetical air deployment of 50 kg of typhus agent before the technology of generating drug resistant strains had been documented [2]. The death toll with drug resistant strains could be much higher. *R. prowazekii* is highly resistant to all but two classes of common antibiotics, namely the tetracyclines and chloramphenicol [3,4]. Recently, *R. prowazekii* was made resistant to rifampin and erythromycin by electroporation of a plasmid containing the resistant gene [5,6]. The creation of tetracycline or chloramphenicol resistant strains, which will be much easier now with the availability of the whole genome sequence of *R. prowazekii,* would create one of the most dangerous threats available for the use of bioterrorists [7].

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R. prowazekii has a monomolecular layer of protein arranged in a periodic tetragonal array on its surface [8]. This crystalline layer, representing 10 to 15% of the total protein mass of the rickettsia, was identified as the immunodominant species-specific surface protein antigen (i.e., outer membrane protein B, OmpB). The OmpB was isolated, purified, and biochemically characterized [9–12]. The earliest and dominant immunological anti-protein responses were directed against Omp B in mice, guinea pigs, rabbits, and humans following infection with *R. prowazekii* [13–17]. It has been shown that purified native typhus OmpB induced strong humoral and cell-mediated immune responses. Protective immunity was elicited by typhus OmpB in guinea pig and mouse protection models [13,15,17]. Outer membrane proteins have also been shown to be the major virulence determinants in other Gram-negative organisms, such as *Aeromonas salmonicida* and *Campylobacter fetus* [18,19], suggesting OmpB may be a good vaccine candidate. Western blot analysis of partially digested OmpB using patient sera revealed that all the reactive fragments were larger than 20 kDa [20]. Among these reactive fragments, one of them was located at the N-terminus (fragment A).

Different reactive fragments, including fragment A, as well as full-length OmpB are known to have posttranslational modifications (i.e., methylation). Previously, we demonstrated that lysine residues were heavily methylated in OmpB from virulent strains but hypo-methylated in those from attenuated strains [9,21] by amino acid composition analysis. In an attempt to generate recombinant reagents that mimic rickettsia derived antigens, we cloned and expressed a recombinant fragment A consisting of amino acid residues 33–272 of the OmpB from *R. prowazekii* (Ap). The *E. coli* expressed Ap was purified, refolded, and then chemically methylated under a vacuum with the volatile reagent CH_3I. Previous approaches using Edman degradation and amino acid composition analysis could not fully characterize the entire protein fragment, especially the sites and level of multiple methylation such as mono-, di- and tri-methylation. In this report, we demonstrated the use of a LC-MS, denovo sequencing and motif pattern recognition software to identify the entire sequence and its posttranslational modification of lysine with methylation. The same approach has also been used for the subsequent characterization of methylation sites in the naturally occurring OmpB.

2. Materials and methods

2.1. Production of recombinant protein Ap

Specific primers for protein Ap (forward: 5′-TCTGGTGTACATATGGGTGCTG(T/C)TATGCAATA-TAATAG-3′, reverse: 5′-ACTGACGGATCCTTATTAAC-CAGTACCGTCT(C/A)TTCCATTAAAAT-3′) were designed and synthesized by Life Technology (Gaithersburg, MD, USA). The genomic DNA of *R. prowazekii* was used as a template in polymerase chain reaction (PCR) to generate the desired fragment Ap. The amplified fragment was ligated into the plasmid pET24a (Novagen, Madison, WI, USA) and was transfected into *E. coli* BL21 (Novagen). Cells were grown in 2YT medium followed by induction of protein expression with 1 mM IPTG (isopropyl B-D thiogalactopyranoside; Sigma-Aldrich, St. Louis, MO, USA) for 3 h. Following centrifugation at 4000 rpm for 20 min, the cell pellet was resuspended in buffer A (20 mM Tris–HCl, pH 8.0, Sigma-Aldrich), containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and disrupted by sonication. The overexpressed recombinant Ap in inclusion body was pelleted and washed sequentially with 2 M urea (Acros, Pittsburgh, PA, USA) and 2% deoxy cholate (Sigma-Aldrich) in Buffer A. The washed inclusion bodies dissolved in 8 M urea were purified by DEAE anion-exchange chromatography with a linear NaCl (Sigma-Aldrich) gradient of 0.70–0.86 M in 8 M urea, 20 mM Tris–HCl, pH 8.0. The peak fractions containing the majority of protein Ap were near homogeneity as determined by SDS-PAGE (Invitrogen, Carlsbad, CA, USA). The N-terminal sequence of Ap was confirmed by Procise 491 protein sequencer (Applied Biosystems, Foster City, CA, USA).

2.2. Refolding of purified recombinant Ap

The purified Ap in 8 M urea was refolded by sequential dialysis in decreasing concentrations of urea as described by Ching et al. [22]. Approximately 0.5 mg/mL of the purified polypeptides in 8 M urea in Tris–HCl buffer was transferred into a dialysis bag (24 mm, MWCO 12,000 Da) and dialyzed sequentially against 6, 4, 2, and 0 M urea in the buffer for 30 min twice at each concentration of urea with gentle stirring. All dialysis procedures except the last step without urea were done at room temperature; usually three dialysis bags with 10 mL each in a 500-mL beaker or 20 mL each in a 1000-mL beaker were dialyzed against buffer at a ratio of 1:15. The dialysis was continued overnight without urea in large excess of buffer A at 4 °C to remove traces of urea.

2.3. Chemical methylation of recombinant Ap

Refolded Ap was lyophilized in 20 mM Tris–HCl, pH 8.0. The lyophilized Ap was used for chemical methylation with CH_3I (Sigma-Aldrich) under vacuum at 37 °C for 18 h as described by Taralp and Kaplan [23].

2.4. Determination of multiple methylation at lysine residues by liquid chromatography-mass spectrometry (LC/MS, ProteomeX)

One milligram each of refolded Ap with or without methylation was denatured in 1 mL of 6 M guanidine

HCl (Sigma-Aldrich), reduced by 10-μL 1 M dithiothreotol (Sigma-Aldrich), alkylated by 25-μL 1 M iodoacetamide (Sigma-Aldrich) at room temperature in the dark. The sample was washed three times in a centricon (10,000 MW cutoff, Millipore, Billerica, MA, USA) with 100 mM ammonia bicarbonate (Sigma-Aldrich). The solution in the final wash was collected and adjusted to a total volume of 1 mL with 100 mM ammonia bicarbonate. The 1 mg/mL of protein solution was digested with 20 μg trypsin (Promega, Madison, WI, USA) overnight at 37 °C. Another 20-μg trypsin was added for an additional 6-h digestion at 37 °C. The digested protein (1 μg) was loaded onto a capillary reversed phased column (75 μm ID×10 cm, Biobasic-C18, ThermoHypersil, Bellefonte, PA, USA) and eluted with a linear gradient of 5–65% acetonitrile (VWR, Bridgeport, NJ, USA) in 60 min. The eluted peptides were introduced into an electrospray ionization, ion trap mass spectrometer (DecaXP Plus or LTQ FT, Thermo Electron, Waltham, MA, USA) for peptide sequence analysis and accurate mass measurement. The SEQUEST search results were initially assessed by examination of the Xcorr (cross correlation) and ΔCn (delta normalized correlation) scores. The Xcorr function measured the similarity of mass-to-charge ratios (m/z) for the fragment ions between the predicted value from published amino acid sequences and the observed value from the MS/MS spectrum. The ΔCn score was obtained by normalizing the Xcorr values to 1.0 and calculating the difference between the first- and second-ranked amino acid sequences [24]. Thus, the ΔCn score discriminated high quality spectra from noisy spectra even when both spectra may have matched a theoretical spectrum. The Sp is the preliminary score assigned after initial comparison between the theoretical generated spectra and the experimental spectra. As a general rule, an Xcorr value of greater than 2.5 for triply, 2.0 for doubly and 1.5 for singly charged ions and a ΔCn greater than 0.1 were accepted as a positive identification [25,26]. Manual inspections of key spectra were performed to confirm the SEQUEST result. Bioworks 3.1 was used to construct a unified ranking score based on the three matching factors (Sp, Xcorr, and ΔCn) [27]. Two additional programs DeNovoX and SALSA for pattern recognition as part of the BioWorks 3.1 package (Thermo Electron, Waltham, MA, USA) were applied for further analysis of the otherwise unidentified spectra and posttranslational modifications.

3. Results

3.1. Purification of recombinant Ap

Production of recombinant Ap was carried out as described in Materials and methods. The recombinant Ap, purified by DEAE anion exchange chromatography, was analyzed by SDS-PAGE (Fig. 1). A single band at approximately 26 kDa stained by Coomassie blue is shown in the insert of Fig. 1.

3.2. Characterization of recombinant Ap before and after methylation

The Ap in the SDS-PAGE gel was transferred onto a PVDF membrane for the subsequent Edman N-terminal amino acid sequence analysis. The results were consistent with the expected sequence (Fig. 4, underlined sequence). The infrared absorption spectra for Ap before and after methylation exhibited significant differences, suggesting that the secondary structure of Ap was changed after methylation (data not shown). Methylated Ap showed higher apparent molecular weight than the non-methylated control as indicated by their electrophoretic mobility in SDS-PAGE (data not shown).

3.3. LC-MS analysis of multiple methylation on lysine residues

The tryptic digests of the methylated and non-methylated Ap were each loaded onto separate capillary C-18 LC columns for LC-MS analysis (see Materials and methods for details). The profiles of ion intensity vs. elution time for methylated and non-methylated Ap are shown in Fig. 2, panels A and B, respectively. The eluted peptides from the reversed phase column were introduced into the ion trap by electrospray ionization. The first full MS scan yielded precursor ions and the top three most abundant precursor

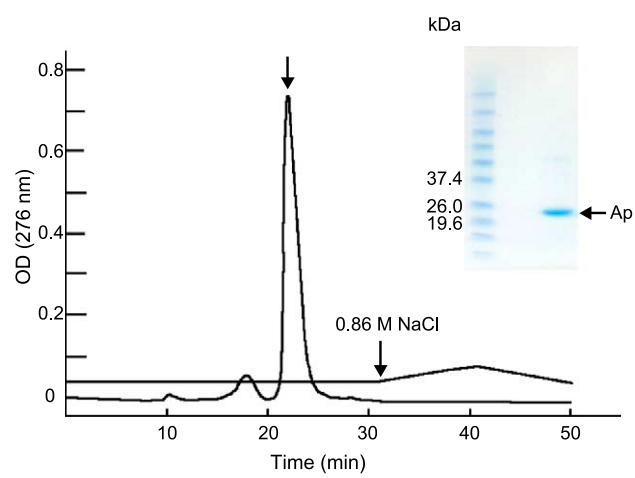

Fig. 1. Chromatogram of recombinant Ap purification. The recombinant Ap was purified by DEAE anion exchange chromatography with Agilent 1100 HPLC. The elution chromatogram is shown as a plot of time (minutes, *X*-axis) vs. absorbance (OD at 276 nm, *Y*-axis). The shallow linear gradient of NaCl from 0.7 to 0.86 M in 30 min was also plotted. The peak fraction (indicated by arrow) eluted from the DEAE anion exchange column was analyzed by SDS-PAGE as shown in the insert.

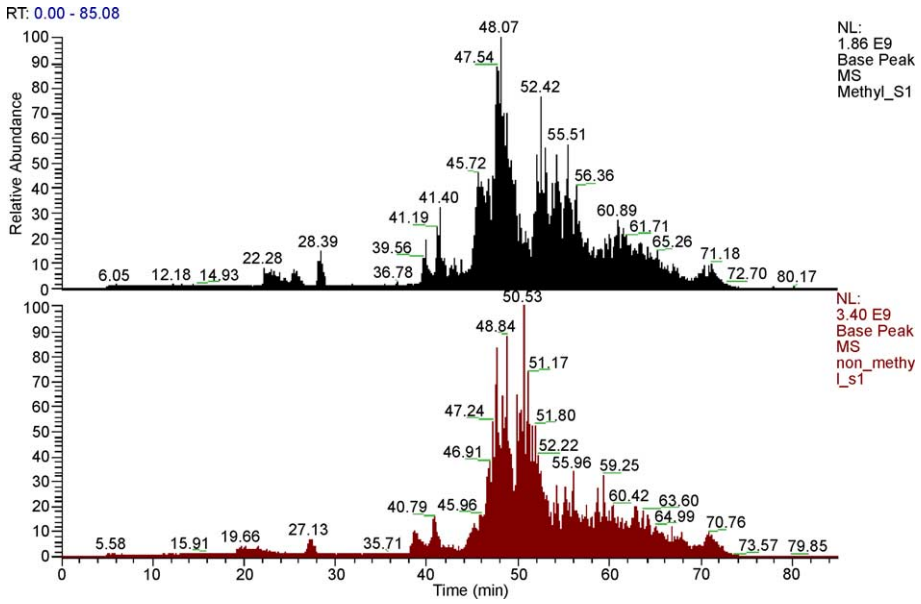

Fig. 2. LC/MS/MS elution profile of methylated (Top) and non-methylated (Bottom) Fragment Ap. The base peak ion abundance (Y-axis) was plotted against time (minutes, X-axis). The analysis details were described in the Materials and methods.

ions were selected for collisional induced fragmentation (set at 35%) to yield sequence information (MS/MS scan). The aforementioned LC-MS analysis relied on a programmed MS/MS fragmentation performed on selected ions. The resulting MS/MS spectra were then matched with predicted spectra from protein databases or the known protein sequence database (e.g., Ap protein sequence). Typically, the ions for MS/MS analysis were selected based on signal intensity. The SEQUEST software automatically assigned MS/MS spectra to peptide sequences or modified peptide sequences (e.g., peptide sequence modified by methylation on lysine residue), calculated a probability for this assignment, and then matched peptides to possible protein identifications [28]. Examples of the methylated (Panel a) and non-methylated (Panel b) peptide assignments are shown in Fig. 3. The peptide sequences were usually fragmented at their amide bond linkage, generating “y” ions and “b” ions (modified Roepstorff and Fohlmann notation). Thus, a series of either “y” or “b” ion matches usually gave a high Xcorr score for the assignment (see Materials and methods for details).

There were two important factors for obtaining the high sequence coverage. First, the enzymatic digestion conditions used should be optimized for complete cleavage. We used a double dose of trypsin in sequential intervals as described in Materials and methods. Often, the hydrophobic domain or the modification at the tryptic cleavage sites rendered an incomplete digestion. However, once the proteins were partially unfolded, the second dose of trypsin was able to further digest the fragmented proteins to yield more complete cleavages. Some nonspecific cleavage may have occurred because of the second dose of trypsin, increasing the complexity added onto the digested peptides. The second important factor was a software package to recognize every possible cleavage in order to compare the experimental MS/MS spectra with the theoretical MS/MS spectra. After intensive search and comparison using SEQUEST, we found that 100% of the sequence of the non-methylated Ap was identified whereas 87.7% of the complete sequence of the methylated Ap (Fig. 4) was identified. In these identified sequences, we found peptides containing mono-, di- and tri-methylated lysines (Fig. 4). The spectra of peptides containing modified lysines were carefully examined manually to assure the assignments were correct. Among the six lysines in the methylated Ap, four of them were mono-, di-, and tri-methylated, one was mono-, and di-methylated and the C-terminus lysine was not observed.

Visual inspection of a MS/MS spectrum from a peptide that was not identified by SEQUEST search using a database containing Ap sequence showed a peptide-like fragmentation character. The presence of unanticipated posttranslational modifications (PTMs) was suspected. Using de novo sequence software (DenovoX), we identified the partial sequence VMQYNR, which was used to research the protein database. This sequence matched a partial sequence of the OmpB from *R. typhi*. As shown in Fig. 5, there was an additional 43 Da unaccounted for even if an extra GA was added to the sequence according to the sequence of full-length OmpB from R. *typhi*. The difference of 43 Da may be accounted for by a carbamylation because of the presence of urea in our protein purification process.

The results of sequence motif search (SALSA) confirmed that the sequence of GAVMQYNR was modified

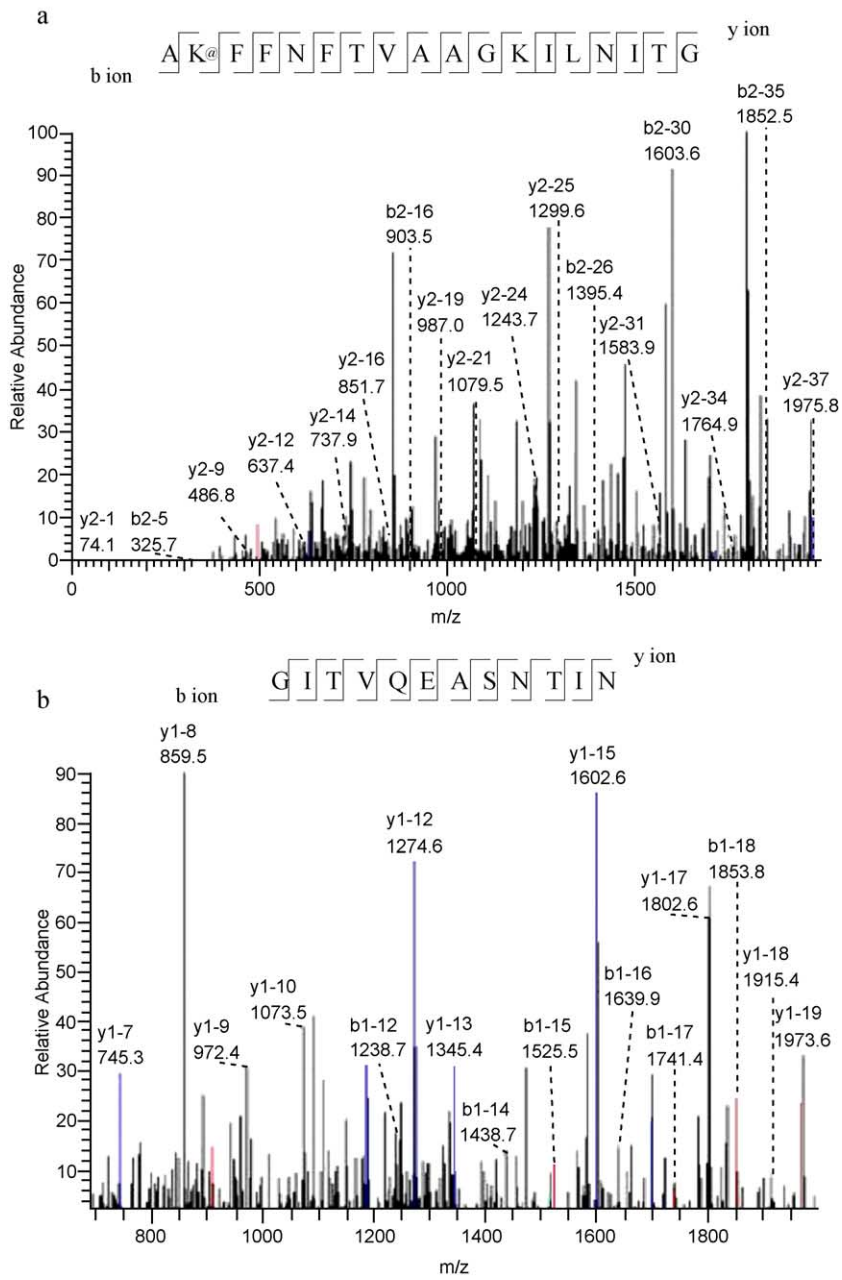

Fig. 3. Example of the MS/MS spectra of the peptide from a doubly charged, methylated peptide from Ap is shown in Panel a. The tri-methylated lysine was labeled with @ and was confirmed by the signal at 1975.8 (*m*/*z*). The singlet charged ion from non-methylated Ap is shown in Panel b; only partial sequence of the identified peptide was shown. The matched "y" and "b" ions are displayed.

(Fig. 6). SALSA searched all MS/MS spectra for those that contain the specified motif and found two doubly charged precursor ions differed by 21.56 Da, suggesting that the difference was due to carbamylation on the N-terminal glycine residue. These results were further evaluated by analyzing the methylated Ap with an FTMS and were

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GAVMQYNRTT NAAATTFDGI GFDQAAGAni pvapnsvita nANNPITFNT
PNGHLNSLFL DTANDLAVTI NEDTTLGFIT NIAQQA*K*FFN FTVAAG**K**ILN
ITGQGITVQE ASNTINAQNA LT**K**VHGGAAI NANDLSGLGS Itfaaapsyl
efnlINPTTQ EAPLTLGANS **K**IVNGGNGTL NITNGFIQYS DNTFAGI**K**TI
NIDDCQGLMF NSTPDAANTL NLQVGGNTIN FNGidgtgk
```

Fig. 4. Tryptic peptides eluted from the LC-MS were identified by BioWorks 3.1. The sequence of fragment Ap is shown with the peptides identified in both methylated (87.7%) and non-methylated (100%) Ap shown in upper case letters. The peptides not identified in non-methylated Ap are in lowercase letters. The lysines with different extents of methylation are shown as k: un, **K**: mono-, di- and tri-methylated lysine, *K*: mono- and di-methylated lysine.

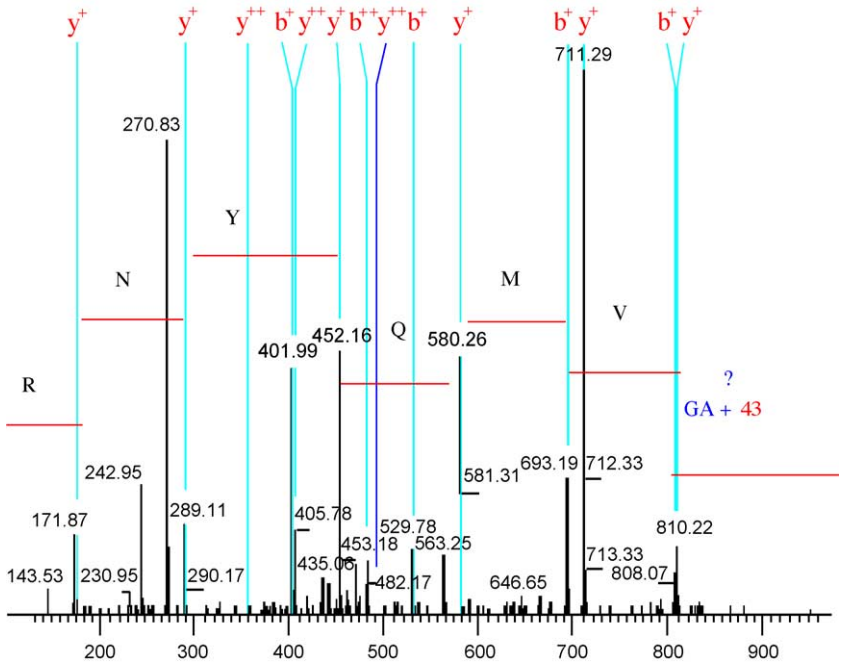

Fig. 5. De novo sequencing by DeNovoX of an unidentified mass spectrum. De novo sequence of a mass spectrum not identified by SEQUEST, indicating an N-terminal modified glycine residue.

confirmed that carbamylation was associated with the N-terminal glycine (data not shown).

4. Discussion

We have previously demonstrated by amino acid composition analysis that OmpB from virulent strains was hypermethylated and that from attenuated strains was hypomethylated [21]. In this report, we attempted to generate recombinant antigens to replace the whole cell antigens currently used in laboratory diagnosis for epidemic typhus. To characterize the chemically methylated recombinant antigen, we not only identified the sites of methylation on recombinant Ap, but also the extent of methylation on each available lysine in recombinant Ap. In addition, this is the first time that a LC-MS technique was used to directly pinpoint the sites of methylation on a recombinant fragment of this outer membrane protein.

A chemically methylated recombinant fragment A from *R. typhi* (At) showed higher reactivity than non-methylated At with patient sera in an enzyme linked immunoabsorbant assay (Ching et al., unpublished results). This suggested the potential use of chemically modified fragments instead of native fragments as antigens in diagnostic test kits or as vaccine components. However, chemical methylation of recombinant proteins may not have the same selectivity as the enzymatic methylation derived in vivo. Nevertheless, the chemical methylation provided a useful platform to study the effect of methylation on the sero-reactivity of fragment At, and the methylated fragment At appeared to maintain the epitopes required to react with antibodies in patients' sera.

Stewart et al. have established a condition where chemical methylation occurred exclusively on lysine residues using small peptides as their model compounds [29]. They further confirmed an exclusive tri-methylation on lysine residues occurred under the experimental condition. We followed similar procedures but the reaction temperature was at 37 °C instead of 72 °C in order to maintain the folded structure of Ap since reaction at 72 °C may denature our expressed protein. In contrast to an exclusive tri-methylation reaction observed at 72 °C, lower temperature at 37 °C may have contributed to the multiple methylation patterns, i.e., addition of mono-, di- or tri-methyl groups on a single lysine (Kaplan, personal communication). However, with the preliminary data on the analysis of lysine methylation of rickettsial derived OmpB, we also found similar complexity of methylation patterns on these lysine residues (data not shown), indicating that the methylation reaction in vivo led to the addition of mono-, di- or tri-methyl groups on a single lysine. These observations suggested that our chemical methylation in vitro at 37 °C mimics the in vivo methylation more closely than the in vitro reaction at 72 °C.

Due to the decreased enzymatic activity of trypsin at trimethylated lysines [30], tryptic digestion of methylated Ap may not have been completed. Some peptides appeared to be quite long because cleavage occurred only at arginine residues (and not lysine residues). Some peptides were not cleaved at either lysine or arginine but at nonspecific sites. The nonspecific cleavage was probably a result of the two sequential trypsin digestions used or minor protease contaminants (i.e., chymotrypsin activity) in the trypsin source. When using SEQUEST to consider all possible cleavages, we found additional peptides that were not identified initially

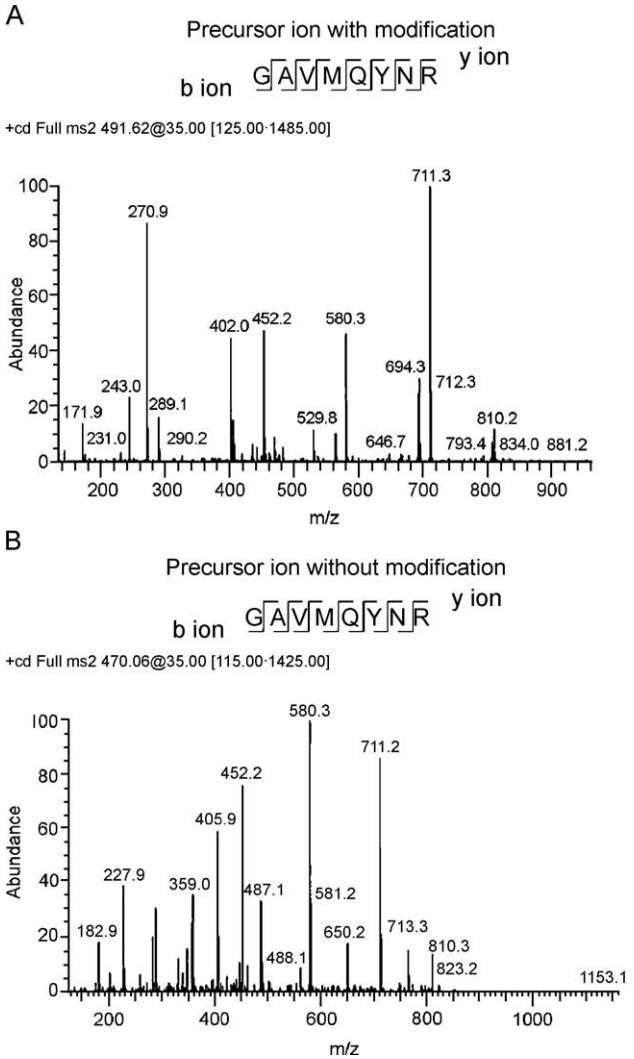

Fig. 6. Use of motif recognition search program (SALSA) to confirm peptides with and without modification. Panel A: MS/MS spectrum of a doubly charged precursor ion (GAVMQYNR) with mass (m/z) 491.62. Panel B: MS/MS spectrum of another doubly charged precursor ion with similar motif (GAVMQYNR) but the precursor ion mass was 470.06. The difference in m/z was 21.56 for a doubly charged ion, indicating a modification on this precursor ion.

when using trypsin specificity as the search parameter. This strategy resulted in 100% and 87.7% sequence coverage for non-methylated and methylated Ap, respectively.

The protein sequencing data using Edman degradation revealed a mutation near the N-terminus where an alanine (GAAMQYNR, *R. prowazekii*) was replaced with a valine (GAVMQYNR, *R. typhi*). It is important to point out that the N-terminal amino acid was not methionine as determined by both the N-terminal protein sequencer and mass spectrometer analysis despite the fact that the expression plasmid was designed with methionine as the N-terminal amino acid. The removal of methionine was probably related to the in vivo protein processing in bacterial expression system. The fact that an alanine was replaced with a valine was due to the forward primer used for polymerase chain reaction (PCR) amplification, which was designed for both organisms.

With the help of computer software (DenovoX), we were able to perform de novo sequence using the MS/MS spectra and determined the sequence of an unknown peptide. This was demonstrated in our search for the N-terminal amino acid residues. Our original search against the database of OmpB from *R. prowazekii* did not identify this N-terminal residue; however, de novo sequencing identified the sequence VMQYNR. This VMQYNR was used for subsequent database searches to identify it as the partial N-terminal sequence of OmpB of *R. typhi* (GAVMQYNR). This verified the advantage of de novo sequencing and further confirmed the sequence identified by protein sequencing.

A pattern recognition software (SALSA) was used to look for a specific motif and specific modifications on the motif [31]. By doing this, we identified a motif (GAVMQYNR) that showed a mass difference of 43.12 Da of two doubly charged precursor ions in the full MS spectra, indicating the possibility of a modification at the N-terminal glycine. Since the protein was purified in the presence of urea, it was possible that N-terminal glycine was carbamylated. A small aliquot of sample was thus analyzed by a Fourier transform mass spectrometer (LTQ FT mass spectrometer) with accurate mass measurement and the results confirmed that the N-terminal glycine was mainly carbamylated (Supplemental Fig. 1). The FTMS data clearly demonstrated the observed isotope distribution of MS spectrum of the 491.62 precursor ion was identical to the theoretical MS spectrum of the carbamylated N-terminal glycine peptide, confirming the N-terminal glycine was carbamylated.

In the above approach using the combination of de novo sequencing (interprets peptide sequences from their MS/MS spectra) and SALSA (detects specific features in MS/MS spectra and scores the spectra based on how many of the features are displayed and their intensities in the spectrum) [31], it was possible to identify peptide sequences that were not identified by SEQUEST. Consequently, we identified a peptide sequence (GAVMQYNR) with and without a carbamylation. Although the carbamylated and non-carbamylated peptides appeared to have similar MS/MS spectra, their precursor ion masses were different.

In conclusion, we have demonstrated the sites and extent of methylation in a chemically methylated recombinant Ap. This chemically methylated protein was used as a model system to study its reactivity toward patient serum for the development of diagnostic tools. We are currently analyzing the variation of methylation in outer membrane proteins from a variety of rickettsiae to better understand the influence of location and extent of methylation of the outer membrane protein on the virulence of rickettsiae.

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not to be construed as official or reflecting the views of the Navy Department, the Army Department or the Department of Defense at large.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2004.08.013.

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