JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. **33**, 1009–1016 (1998)

Identification of the Active Site Serine of Penicillin-binding Protein 2a from Methicillin-resistant *Staphylococcus aureus* by Electrospray Mass Spectrometry

Yiping Sun,[1]* Mark D. Bauer[1] and Weiping Lu[2]
[1] Corporate Research Division, The Procter & Gamble Co., Miami Valley Laboratories, P.O. Box 538707, Cincinnati, Ohio 45253-8707, USA
[2] P & G Pharmaceuticals, Health Care Research Center, 8700 Mason-Montgomery Road, Mason, Ohio 45040, USA

Penicillin-binding protein 2a (PBP2a), a high molecular mass PBP, is the primary enzyme responsible for the β-lactam resistance in methicillin-resistant *Staphylococcus aureus* (MRSA). Inhibition of a PBP such as PBP2a by β-lactams is due to covalent modification of an active site serine residue. Based on the sequence alignment with well studied β-lactamases, DD-carboxypeptidases and other high molecular mass PBPs, the serine of a tetrad S_{403}XXK in PBP2a was tentatively identified as the penicillin-binding site. However, direct evidence for the involvement of serine403 has not been reported. In this study, a method which combines liquid chromatography/electrospray mass spectrometry (LC/MS) and nano-electrospray MS for the identification of the active site serine in PBP2a is described. The covalent binding of the β-lactams was carried out *in vitro* with the recombinant PBP2a. Peptide mapping of the cyanogen bromide fragments from penicilloyl-PBP2a, using microbore LC/MS, provided a rapid identification of the modified peptide with a 334 Da mass increase. The acylated peptide was isolated and further digested with trypsin. Nano-electrospray MS/MS sequencing of the acylated peptide in the tryptic digest showed that the penicillin was indeed attached to serine403. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: penicillin-binding protein 2a; active site serine; liquid chromatography/mass spectrometry; nano-electrospray mass spectrometry

INTRODUCTION

High molecular mass (HMM) penicillin-binding proteins (PBPs) are the enzymes responsible for the final stages of the bacterial peptidoglycan synthesis and are the lethal targets of β-lactam antibiotics.[1] Resistance to β-lactams in methicillin-resistant *Staphylococcus aureus* (MRSA) is primarily due to the production of a novel low β-lactam affinity penicillin-binding protein, PBP2a or PBP2′, which can apparently substitute the functions of the normal, sensitive PBPs when they are inactivated by methicillin or other β-lactams.[2] It is generally accepted that irreversible inhibition of PBPs by β-lactams is due to the formation of a stable covalent acyl-enzyme adduct involving an active site serine residue.[2] Based on amino acid sequence alignments with well studied β-lactamases, a DD-carboxypeptidase and other HMM PBPs, the serine of a tetrad S_{403}XXK in the amino acid sequence of PBP2a was tentatively identified as the penicillin-binding site.[3] Recombinant, soluble PBP2a has been purified and characterized in several laboratories.[4–7] However, an effort to obtain direct evidence for the involvement of serine403 in the active site failed.[8]

Electrospray mass spectrometry is a powerful analytical technique for the structural characterization of proteins. Many applications, including verification of the expression of the correct protein sequence, identification of post-translational modifications, location of inhibitor-binding sites and characterization of non-covalent complexes of receptor–ligand and enzyme–substrate, have been reported.[9–11] Peptide mapping of the active site of *Bacillus cereus* β-lactamase I by the use of high-performance liquid chromatography (HPLC) couple with electrospray mass spectrometry has been described.[12] More recently, nano-electrospray mass spectrometry has been demonstrated for sequencing peptides in complex mixtures using minute samples and for identifying proteins isolated by 1D and 2D gel electrophoresis.[13–15]

In this study, an approach which combined chemical/enzyme digestion, microbore liquid chromatography/electrospray mass spectrometry (mLC/MS), perfusion capillary LC/MS (pcLC/MS) and nano-electrospray (nES) MS was used to identify the active site serine in

* *Correspondence to*: Y. Sun, Corporate Research Division, The Procter & Gamble Co., Miami Valley Laboratories, P.O. Box 538707, Cincinnati, Ohio 45253-8707, USA
E-mail: suny.@pg.com

CCC 1076–5174/98/101009–08 $17.50
© 1998 John Wiley & Sons, Ltd.

Received 3 April 1998
Accepted 9 July 1998

PBP2a acylated with benzylpenicillin. The results indicated that the serine of the $S_{403}XXK$ motif in PBP2a was indeed the residue that was acylated by benzylpenicillin. For the first time, direct evidence for the role of serine403 in HMM PBPs has been obtained.

EXPERIMENTAL

Chemicals and reagents

Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, methicillin and ampicillin were purchased from Sigma (St Louis, MO, USA), cyanogen bromide (CNBr) and trifluoroacetic acid (TFA) from Aldrich (Milwaukee, WI, USA), formic acid (88%) and acetonitrile from Baker (Phillipsburg, NJ, USA) and benzylpenicillin from Fluka (Ronkonkoma, NY, USA).

Cloning, expression and purification of PBP2a

Detailed cloning, expression and purification of a soluble PBP2a will be described elsewhere.[16] Briefly, chromosomal DNA of an MRSA clinical isolate (strain MI339) obtained from the Public Health Research Institute of New York City was used as the template for PCR. In the forward primer an *Nco*I restriction site was introduced before the codon for Ala24, resulting in the *mecA* gene coding for a truncated PBP2a, of which the membrane-spanning segment consisting of the first 23 amino acids at the *N*-terminus was deleted. The resultant plasmid pTMCA was transferred into an *E. coli* expression strain BL21/DE3 (Novagen) and overexpressed following the manufacturer's protocol. PBP2a in cell-free extract was purified to homogeneity in large quantity (50–100 mg) using chromatography with Q-Sepharose, Q-15 Phenyl-Sepharose and Superdex 75 columns.

Acylation of PBP2a

About 10–15 μg of PBP2a were incubated with 0.7 mM benzylpenicillin for 2 min, 1 mM ampicillin for 3 min and 1 mM methicillin for 15 min in a final volume of 100 μl of 10 mM phosphate buffer containing 50 mM NaCl (pH 7.2). The reaction was carried out at 37 °C and terminated by adding 30 μl of 5% HCOOH. The mixture was then transferred into an Amicon Microcon 30 tube to remove the free β-lactam and most of the salts. Centrifugation was performed at 11 000*g* for 5 min at room-temperature. The recovered protein solution was collected and its volume was adjusted to ~25 μl with 1% HCOOH. Each acylated PBP2a was analyzed by pcLC/MS.

Chemical cleavage and enzymatic digestion

PBP2a or penicilloyl-PBP2a (about 0.1 mg each) was purified by HPLC. CNBr cleavage of the protein was carried out in 0.1 M HCl for 2 h at 37 °C in the dark. The CNBr fragment of interest was isolated by HPLC and further treated with trypsin in 0.1 M NH_4OAc (pH 8.5) for 30 min at 37 °C. The tryptic digest was analyzed by both mLC/MS and nES/MS/MS.

Liquid chromatography mass spectrometry

All electrospray MS analyses were carried out on a Perkin–Elmer SCIEX (Thornhill, ON, Canada) API-III LC/MS/MS triple quadrupole instrument. The instrument was scanned over a mass range of 300–2400 mu using a 1.0 ms dwell time and 0.2 or 0.4 mu step size. The instrument was calibrated using polypropylene glycol (PPG), with a typical mass accuracy of better than 0.01%. On-line mLC/MS was carried out on a Waters 600-MS system. A pre-injector split ratio of 9.5:0.5 was established by splitting one side to a 'dummy' column (Waters Delta Pak C_{18}, 3.9 × 150 mm) and splitting the other side to a 1 × 150 mm C_4 microbore column (Vydac, 300 Å, 5 u). A flow-rate of about 50 μl min^{-1} was achieved. A Gilson Model 234 auto-injector with sample cooling was used. The mobile phase solvents were 0.02% TFA in H_2O (solvent A) and 0.02% TFA in CH_3CN (solvent B). A gradient of 1–85% B over 40 min was used. For pcLC/MS, the same set-up as for mLC/MS was used except that the column was an R2/H Poros column (0.3 × 5 or 10 cm) (LC Packings, San Francisco, CA, USA). A gradient of 1–85% B over 5 or 10 min was used.

nES/MS/MS was carried out on the same instrument with a nano-electrospray source designed by Wilm and Mann[13] (European Molecular Biology Laboratory, Germany). One millimeter gold-coated, pulled glass capillaries were purchased from Protein Analysis (Denmark). The capillary was loaded on to the holder. A positive pressure was applied to the holder via a gas-tight syringe. A potential of 700–800 V was applied to the needle, 100 V to the interface plate and 60–80 V to the orifice. The needle was positioned directly in front of the orifice. Prior to positioning, the spray needle was gently touched on the interface plate to initiate flow. Typically, the electrospray signal from 1–2 μl of a sample solution lasted about 30 min. The long signal duration in nES/MS/MS allowed the sequencing of peptides with weak signals. Argon was used at the collision gas. The collision gas thickness was (100–200) × 10^{12} atoms $cm.^{-2}$

RESULTS AND DISCUSSION

Sequence verification of the recombinant PBP2a

Recombinant PBP2a, with a deletion of the first 23 amino acids at the *N*-terminus, has 645 amino acids, containing 16 methionines and no cysteines (Fig. 1). According to DNA sequence analysis, the *mecA* gene (pTMCA-23) cloned from MRSA strain MI339 differed from that of strain 27r by one amino acid (glycine instead of glutamic acid) at position 246 and from that of strain TK784 by 24 amino acids.[16] The purified PBP2a was analyzed by pcLC/MS to determine the

© 1998 John Wiley & Sons, Ltd.

24
ASKDKEINNT IDAIEDKNFK QVYKDSSYIS KSDNGEVEMT

ERPIKIYNSL GVKDINIQDR KIKKVSKNKK RVDAQYKIKT

NYGNIDRNVQ FNFVKEDGMW KLDWDHSVII PGMQKDQSIH

IENLKSERGK ILDRNNVELA NTGTAYEIGI VPKNVSKKDY

KAIAKELSIS EDYIKQQMDQ NWVQDDTFVP LKTVKKMDEY

LSDFAKKFHL TTNETESRNY PLGKATSHLL GYVGPINSEE

LKQKEYKGYK DDAVIGKKGL EKLYDKKLQH EDGYRVTIVD

DNSNTIAHTL IEKKKKDGKD IQLTIDAKVQ KSIYNNMKND

YGSGTAIHPQ TGELLALVST PSYDVYPFMY GMSNEEYNKL

TEDKKEPLLN KFQITTSPG<u>S</u> TQKILTAMIG LNNKTLDDKT
403

SYKIDGKGWQ KDKSWGGYNV TRYEVVNGNI DLKQAIESSD

NIFFARVALE LGSKKFEKGM KKLGVGEDIP SDYPFYNAQI

SNKNLDNEIL LADSGYGQGE ILINPVQILS IYSALENNGN

INAPHLLKDT KNKVWKKNII SKENINLLTD GMQQVVNKTH

KEDIYRSYAN LIGKSGTAEL KMKQGETGRQ IGWFISYDKD

NPNMMMAINV KDVQDKGMAS YNAKISGKVY DELYENGNKK

YDIDE
668

Figure 1. The amino acid sequence of PBP2a. The membrane-spanning segment, consisting of the first 23 amino acids at the *N*-terminus, was deleted from the sequence. The *N*-terminal sequence was confirmed by Edman degradation. The active site residue (Ser403) is underlined.

molecular mass of the intact protein. As shown in Fig. 2(a) and (b), the measured molecular mass was 73 466 Da, which matched very well with the calculated value (73 467 Da) from the predicted sequence. A mass accuracy of better than 0.01% can be routinely obtained on the Perkin-Elmer SCIEX API-III instrument using PPG as the calibrant. To verify the entire sequence, peptide mapping was performed. Cyanogen bromide can specifically cleave at the *C*-termini of methionine residues to generate peptides with homoserine lactones at the *C*-termini. A CNBr-treated PBP2a sample was analyzed by mLC/MS. Fragments identified in the digest are listed in Table 1. The results show complete coverage of the sequence. The *N*-terminal sequence of the intact PBP2a was also determined by Edman degradation.

Detection of PBP2a acylation with β-lactams

The binding of PBP2a with β-lactams such as benzylpenicillin (M_r 334), ampicillin (M_r 349) and methicillin (M_r 380) was evaluated. Because of the high salt buffer used for sample preparation, it was difficult to analyze these sample by direct flow-injection MS. pcLC/MS provides a rapid and reliable method for detecting the modified and unmodified PBP2a by removing salts, buffers and unreacted β-lactams. The modified and unmodified PBP2a were not chromatographically resolved. Figure 3(a), (b) and (c) are the reconstructed mass spectra from the pcLC/MS analyses of PBP2a samples incubated with benzylpenicillin (0.7 mM for 2 min), ampicillin (1 mM for 3 min) and methicillin (1 mM for 15 min), respectively. New peaks at 73 801, 73 816

© 1998 John Wiley & Sons, Ltd.

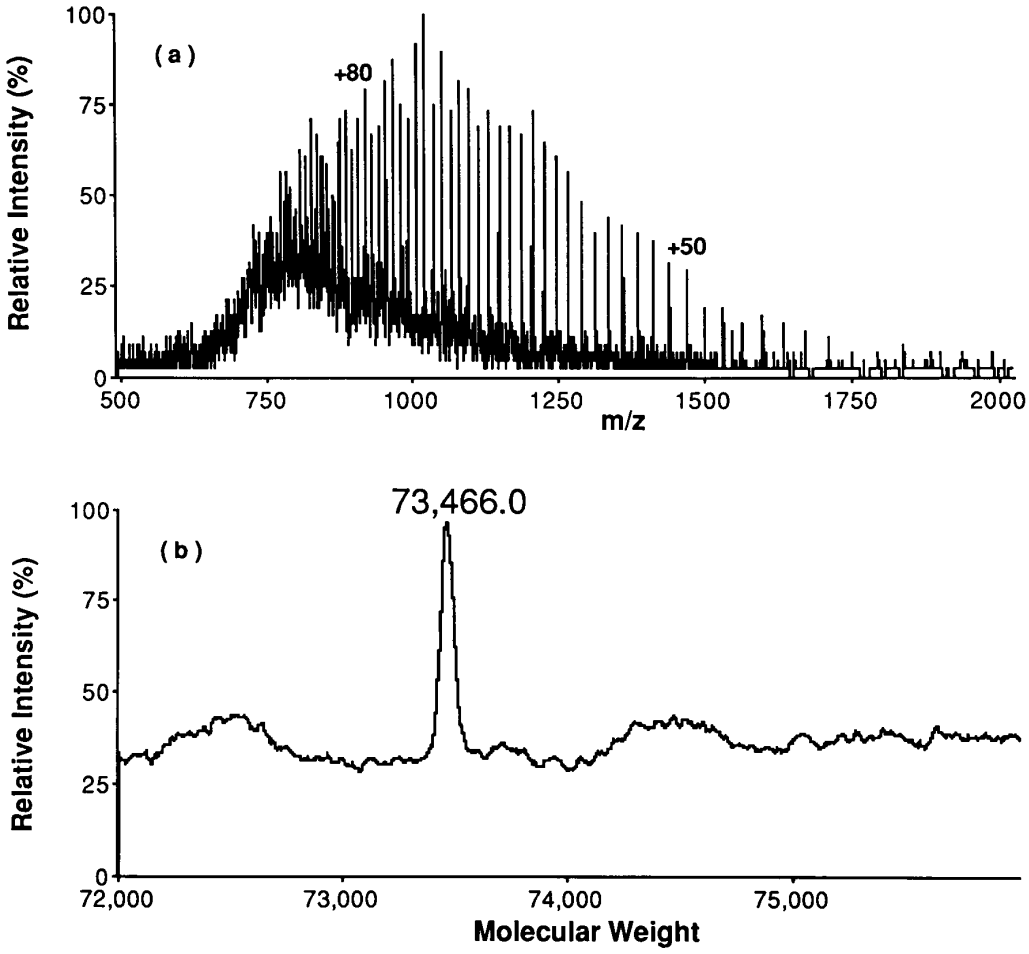

Figure 2. (a) Mass spectrum of the recombinant PBP2a obtained by pcLC/MS and (b) the reconstructed mass spectrum showing the measured molecular mass of 73 466 Da.

Table 1. Assignment of peptides from *E. coli* PBP2a treated with CNBr for 2 h

Peptide[a]	Expected MH^+	Observed MH^+
373–375	322.4	322.2
630–641	1270.4	1269.8
123–136	1649.9	1649.4
202–220	2245.6	2245.2
606–627	2537.8	2537.5
642–668	3128.4	3127.9
576–605	3375.8	3375.5
341–372	3425.8	3425.4
376–411	4051.6	4052.0
24–62	4407.8	4408.1
63–122	7054.1	7054.0
137–201	7358.3	7358.1
412–483	8084.1	8083.9
484–575	10 166.5	10 167.9
24–122	11 460.9	11 460.9
221–340	13 720.5	13 720.5

[a] Average masses are shown. The membrane-spanning segment consisting of the 23 amino acid residues at the *N*-terminus was removed during cloning.

and 73 847 Da were detected, corresponding to the respective acylated PBP2a. All three β-lactams were covalently bound to PBP2a with a ratio of 1:1. The extent of acylation by each β-lactam was measured to be 48.5% for penicillin, 90.0% for ampicillin and 37.8% for methicillin, based on the ratio of peak intensity of the acyl-PBP2a to the total PBP2a. It should be pointed out that the measurement of the percentage of acylation for each β-lactam from the peak intensity was based on the assumption that the ES/MS response of the unmodified and modified PBP2a was the same. Using the pcLC/MS method, the kinetics of PBP2a acylation by a certain β-lactam can be measured. The results of such a study for penicillin binding to PBP2a will be published elsewhere.[16] Furthermore, the method can be used for the rapid evaluation of the activity of PBP2a prepared in different batches.

Location of the active site serine in PBP2a

Identification of the active site of *Bacillus cereus* β-lactamase I (291 amino acids) has been reported using

© 1998 John Wiley & Sons, Ltd.

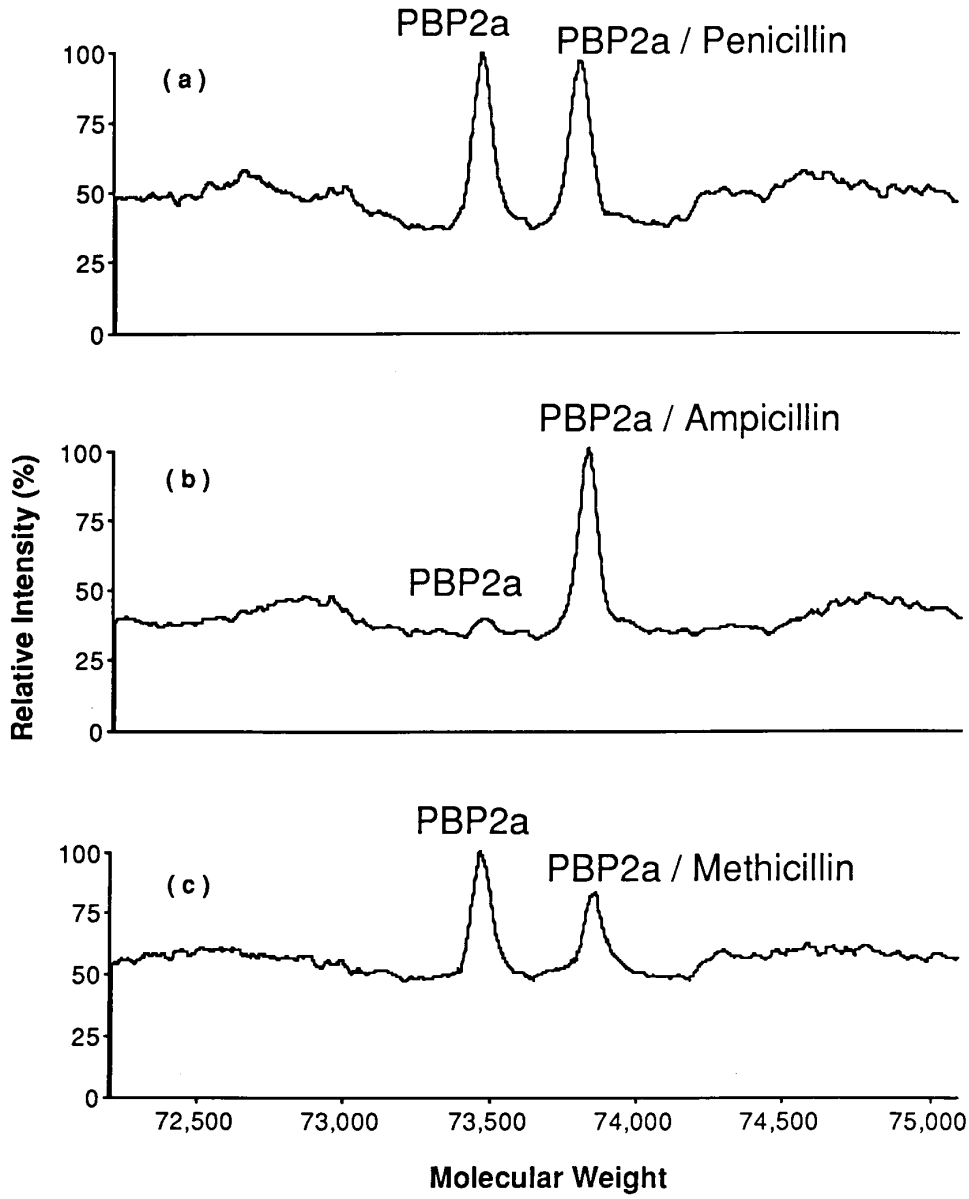

Figure 3. Reconstructed mass spectra showing the inhibition of PBP2a by (a) benzylpenicillin (0.7 mM for 2 min), (b) ampicillin (1 mM for 3 min) and (c) methicillin (1 mM for 15 min). The extent of acylation by each lactam was measured to be 48.5% for benzylpenicillin, 90.0% for ampicillin and 37.8% for methicillin, based on the ratio of peak intensity of the acyl-PBP2a to the total PBP2a.

LC/MS.[12] Attempts to locate the active site serine in PBP2a failed.[8] In this study, a strategy which combined chemical/enzymatic digestion, mLC/MS and nES/MS/MS was used to locate the active site in PBP2a. The strategy is outlined in Fig. 4. After digestion of penicilloyl-PBP2a with CNBr, a large peptide (*m/z* 4386.0, residues 376–411), with the tetrad $S_{403}XXK$ and a 334 Da mass increase, was identified using mLC/MS [Fig. 5(a) and (b)]. The abundant ion (*m/z* 825.6), next to the + 5 ion of the peptide 376–411, was the doubly charged ion of the peptide 123–136 (*m/z* 1649.4).

Although the separation of peptide fragments could be improved, mLC/MS allowed us to identify rapidly the acylated peptide in the mixture. The mass list of the expected CNBr fragments of PBP2a was generated using the BioToolBox software from Perkin–Elmer SCIEX and compared with the peaks measured for peptide identification. A quick ion extraction of the acylated peptide (*m/z* 4386) also allowed us to identify it in

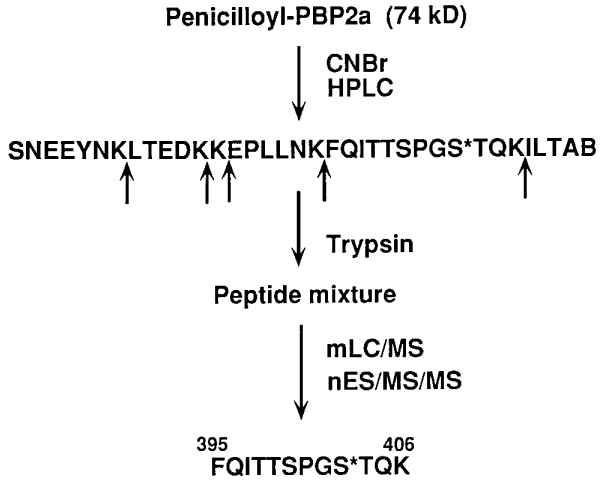

Figure 4. Strategy for locating the penicillin-binding site in PBP2a.

© 1998 John Wiley & Sons, Ltd.

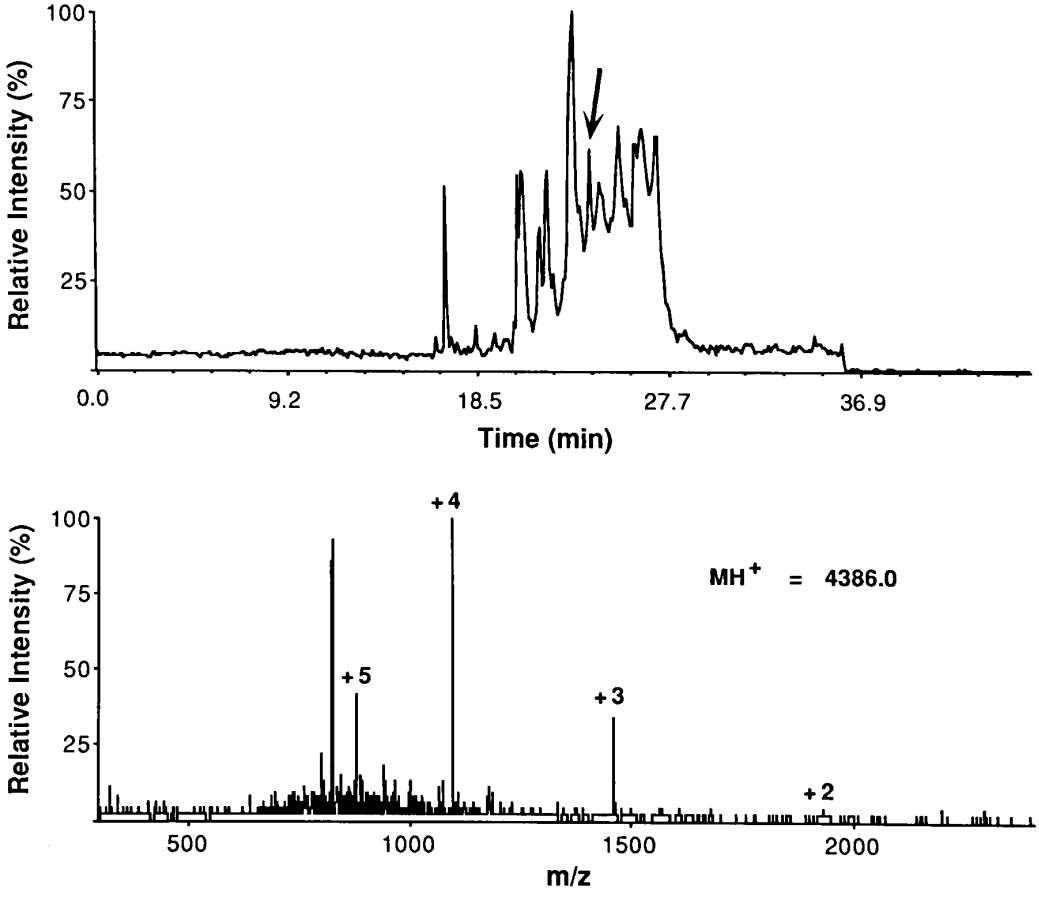

Figure 5. (a) Total ion current trace of the CNBr-treated penicilloyl-PBP2a obtained by mLC/MS. The acylated peptide (residues 376–411) with a retention time of 23.9 min is indicated by an arrow. (b) Mass spectrum of the acylated peptide ($MH^+ = 4386.0$).

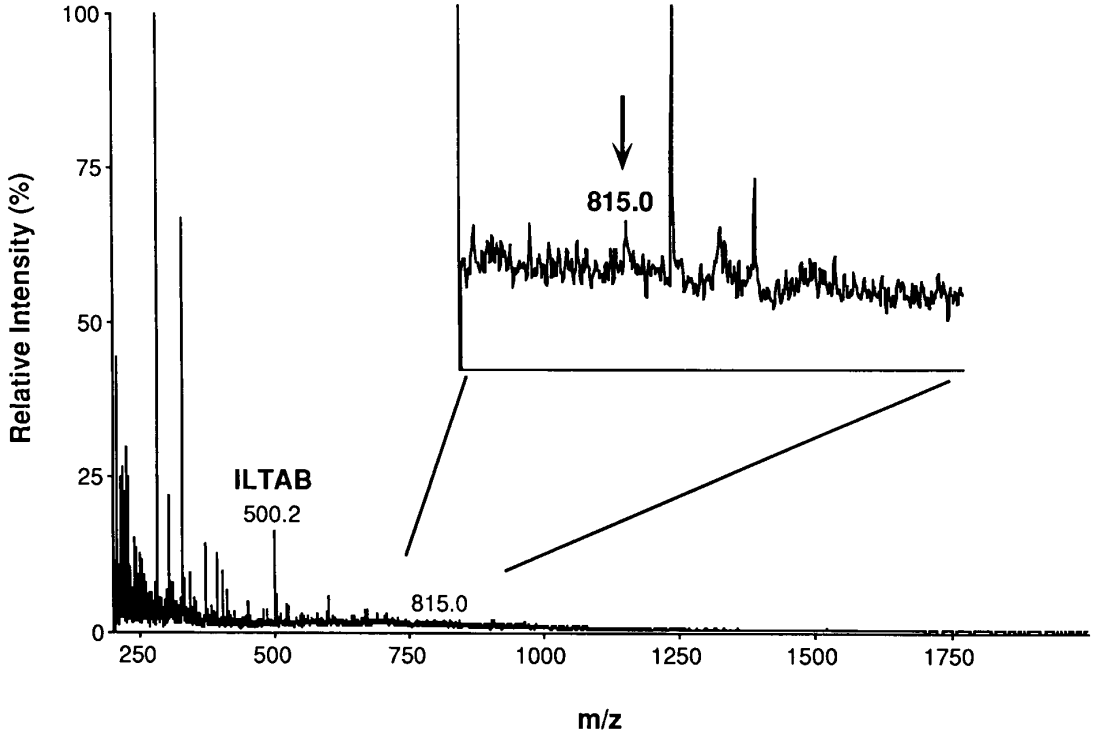

Figure 6. Nano-electrospray mass spectrum of the tryptic digest of the acylated peptide with the retention time of 23.9 min shown in Fig. 5.

© 1998 John Wiley & Sons, Ltd.

the mLC/MS run. After the mLC/MS analysis, the modified peptide (376–411) was isolated from the digest by HPLC. Upon further digestion of the isolate with trypsin, followed by on-line mLC/MS analysis, the expected peptide (FQITTSPGS$_{403}$TQK, *m/z* 1629) with the benzylpenicillin attached was detected (data not shown); however, the peak was weak, indicating that the acylation product was unstable during enzymatic digestion at pH 8.6. This observation was consistent with the results from stability studies in which the acylated PBP2a was hydrolyzed over a period of time at basic pH (data not shown).[16] In an attempt to obtain MS/MS sequencing data, the tryptic digest of the CNBr cleavage isolate was analyzed by nES/MS/MS. nES/MS allows one to analyze complex digestion mixtures using a minute sample volume.[13] In our experiment, both nES/MS and nES/MS/MS data were acquired from a 2 μl sample loading. The nES/MS signal lasted longer than 30 min which allowed signal averaging for improved spectral quality from weak peaks. As shown in the inset in Fig. 6, the nES mass spectrum gave a weak peak at *m/z* 815.0, corresponding to the doubly charged ion of the acylated peptide. Because there were two serine residues in the peptide, nES/MS/MS sequencing of the peptide was necessary to locate the exact site of the modification. Figure 7 shows the nES/tandem mass spectrum of the peak at *m/z* 815.0. *y*-Type ions were predominant in the spectrum owing to the basic residue lysine at the *C*-terminus. A series of fragment ions (y10, y9, y8, y7, y6 and y4), corresponding to the loss of FQ, I, T, T, S and PG, respectively from the *N*-terminus of the peptide, was observed. The peaks at *m/z* 798 (y4, S(pen)TQK), *m/z* 952 (y6, PGS(pen)TQK) and *m/z* 678 (b6, FQITTS) clearly indicate that the penicillin modification site is serine403. The peak at *m/z* 1295.2 came from the loss of the bound penicillin from the acylated peptide. It is worth mentioning that some ions below the doubly charged precursor ion might arise from the background ion due to a weak precursor ion. As shown in Fig. 7, peaks labeled with an asterisk were from the NH_4OAc cluster ions. These cluster ions could be reduced by first drying the digestion buffer solution and re-dissolving it in CH_3OH–H_2O (50:50, v/v) with 0.1% HCOOH for the nES/MS/MS analysis.

CONCLUSIONS

Using the combination of on-line LC/MS and nES/MS/MS, we have identified the exact site of penicillin modification to PBP2a. For the first time, such direct evidence for the role of serine403 in HMM PBP2a has been obtained. mLC/ES/MS is a useful technique for the rapid peptide mapping of proteins. Sequence verification of a large protein and identification of a covalently modified peptide in a digestion can be done quickly by using this on-line technique. pcLC/MS allows one to determine the extent of inhibition of PBP2a by different inhibitors and to study the kinetics of PBP2a binding. nES/MS/MS is a powerful technique for sequencing peptides in complex mixtures. Because signals in nano-electrospray last much longer than those in conventional electrospray, weak peaks from peptides can also be sequenced.[15]

Acknowledgements

The authors thank Dr Feng Wang for the *N*-terminal sequencing of PBP2a and Dr Thomas W. Keough for helpful discussions.

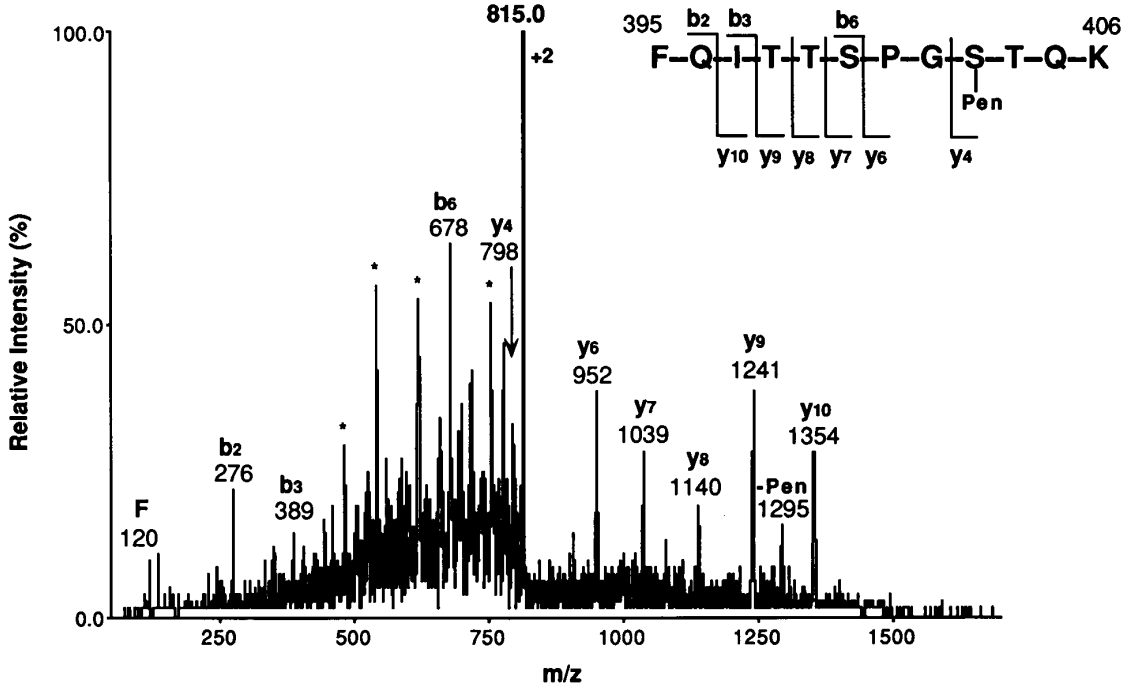

Figure 7. Nano-electrospray tandem mass spectrum of the peak at *m/z* 815.0 shown in Fig. 6. The peak at *m/z* 815.0 corresponds to the doubly charged ion of the acylated peptide (*m/z* 1629.0). The penicillin-binding site was located at serine403. Peaks with an asterisk are from the cluster ions of NH_4OAc used as the digestion buffer.

© 1998 John Wiley & Sons, Ltd.

REFERENCES

1. D. J. Waxman and J. L. Strominger, *Annu. Rev. Biochem.* **52**, 825 (1983).
2. B. G. Spratt, in *Bacterial Cell Wall*, edited by J.-M. Ghusyen and R. Hakenbeck, p. 517. Elsevier, Amsterdam (1994).
3. J.-M. Ghuysen and G. Dive, in *Bacterial Cell Wall*, edited by J.-M. Ghusyen and R. Hakenbeck, p. 103. Elsevier, Amsterdam (1994).
4. C. Y. E. Wu, J. Hoskins, L. C. Balszczak, D. A. Preston and P. L. Skatrud, *Antimicrob. Agents Chemother.* **36**, 533 (1992).
5. L. J. Frank, D. Wisniewski, G. G. Hammond, J. Hermes, A. Marcy and P. M. Cameron, *Protein Expression Purif.* **6**, 671 (1995).
6. S. Roychoudhury, J. E. Dotzlaf, S. Ghag and W. K. Yeh, *J. Biol. Chem.* **269**, 12067 (1994).
7. J. H. Toney, G. G. Hammond, B. Leiting, K. A. D. Pryor, J. K. Wu, G. C. Cuca and D. L. Pompliano, *Anal. Biochem.* **255**, 850 (1998).
8. S. Roychoudhury, R. E. Kaiser, D. N. Brems and W. K. Yeh, *Antimicrob. Agents Chemother.* **40**, 2075 (1996).
9. C. N. McEwen and B. S. Larsen (Eds), *Mass Spectrometry of Biological Materials*. Marcel Dekker, New York (1990).
10. J. Chapman (Ed.), *Protein and Peptide Analysis by Mass Spectrometry*, Humana Press, New York (1996).
11. D. R. Marshak (Ed.), *Techniques in Protein Chemistry VIII*. Academic Press, San Diego (1997).
12. P. L. Roach, J. E. Baldwin, R. T. Aplin, C. V. Robinson and C. J. Schofield, *J. Chem. Soc., Chem. Commun.* 121 (1993).
13. M. S. Wilm and M. Mann, *Int. J. Mass Spectrom. Ion Processes* **136**, 167 (1994).
14. M. S. Wilm and M. Mann, *Anal. Chem.* **68**, 1 (1996).
15. A. Shevehenke, M. S. Wilm, O. Vorm and M. Mann, *Anal. Chem.* **68**, 850 (1996).
16. W.-P. Lu, Y. Sun, M. D. Bauer, S. Paula, P. M. Koenigs and W. G. Kraft, in preparation.

© 1998 John Wiley & Sons, Ltd.