Molecular Heterogeneity of Urinary Proteins in Wild House Mouse Populations

Duncan H. L. Robertson,† Jane L. Hurst, Mark S. Bolgar, Simon J. Gaskell and Robert J. Beynon

1Department of Biochemistry and Applied Molecular Biology, UMIST, PO Box 88, Manchester, M60 1QD, UK
2Behaviour and Ecology Research Group, Department of Life Science, University of Nottingham, Nottingham, NG7 2RD, UK
3Michael Barber Centre for Mass Spectrometry, UMIST, PO Box 88, Manchester, M60 1QD, UK

Major urinary proteins (MUPs) from the urine of individual wild mice were characterized using electrospray ionization mass spectrometry (ESI-MS) and compared to MUPs from the urine of inbred mice. The wild mice showed considerable variation between individuals in the expression of a group of MUPs with similar masses. Some individuals excreted MUPs of unique molecular mass whilst some failed to express MUPs seen commonly in the other individuals. All the wild individuals contained proteins not previously observed in inbred mice. Urine from one individual was fractionated using anion exchange chromatography prior to analysis by ESI-MS. By analysing urine from inbred samples under the same conditions it was possible to relate, using mass and net charge in solution, MUPs from the wild sample to the MUPs that have been observed previously in inbred strains. This has allowed tentative identification of some MUPs from the wild mouse. The effect of collection history of urine from wild mice was also investigated. ESI-MS analysis of MUPs in a faecally contaminated sample showed the loss of a C-terminal tripeptide when compared to an uncontaminated sample from the same mouse, consistent with the presence of a specific endopeptidase. Similarly a sample of pooled urine provided by twelve individuals trapped from the same population showed evidence of loss of the C-terminal dipeptide. © 1997 by John Wiley & Sons, Ltd.

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Mouse urine contains large quantities of protein, predominantly in the form of a group of 18–19 kDa proteins of the lipocalin family, called major urinary proteins (MUPs).1,2 In the wild, urine is deposited in discrete scent marks around a mouse’s territory3 and carries information concerning individual identity, sex, social and reproductive status, and kinship.4,5 The proteins are known to bind volatile, low-molecular-mass ligands which have been demonstrated to be behaviourally active,6 leading to the supposition that MUPs may be involved in the slow release of such molecules and thus mediate an olfactory signal.7

The MUPs are a heterogeneous mixture of proteins that appear as a single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) but which resolve into multiple components on ion-exchange chromatography or isoelectric focusing.8 We have previously demonstrated that electrospray mass spectrometry (ESI-MS), particularly with maximum entropy enhancement of resolution, can be used to ‘fingerprint’ a total MUP preparation derived from inbred mouse strains.9 Moreover, a combination of ion-exchange chromatography and ESI-MS affords even greater resolution, particularly of isobaric charge variants of MUPs.10 From such analyses and from information derived from full or partial cDNA sequences, we have identified at least fourteen different MUP variants in a limited range of inbred mouse strains.11

Although inbred mouse strains facilitate acquisition and analysis of genetically homogenous (and therefore reproducible) material, they are derived from inbreeding programmes over 60 years and therefore represent a rather selective ‘snapshot’ of the wild population. The pattern of MUP inheritance is complex, leading to speculation that the pool of MUP phenotypes in the wild could be highly variable.10 To test this hypothesis, we have embarked upon an analysis of MUP expression in wild mice, collected from different populations and environments.

MATERIALS AND METHODS

Animals and sample collection

Inbred strains were housed as described previously,9 the urine samples analysed from these animals were pools from 5–20 individuals. Wild subjects were 20 adult male mice (Mus domesticus) captured from 5 different populations around the UK and housed individually in cages or enclosures. All were caught from different sites and were unlikely to be close kin. To differentiate wild from inbred samples some wild mice have been coded in the following manner: W-XY, where W denotes an animal of wild origin, X denotes the population from which it was taken, and YY identifies the individual animal. Two of the subjects were second generation offspring of wild-caught mice while 12 originated from an isolated population on the Isle of May. Urine, evacuated when mice were held by the scruff of the neck, was collected into separate glass vials and stored at −18°C. Additional material was added to the frozen samples over a 4 day period. Urine samples from the 12 Isle of May mice were pooled into a single vial.
Sample preparation

Unfractionated MUPs. Aliquots of urine (50–100 μL) were desalted using 5 mL spin Sephadex-G25 columns previously equilibrated in filtered and deionized water (Milli-Q, Millipore, Hertfordshire, UK). MUPs loaded onto such columns were subsequently eluted by centrifugation at 200 g for 1 min. A 10 μL aliquot of each desalted sample was then diluted 1:5 with an aqueous mixture of 50% (v/v) acetonitrile (HPLC grade) + 0.2% (v/v) formic acid prior to analysis by ESI-MS.

Fractionated MUPs. Two aliquots (125 μL) of urine from a given individual mouse or pooled sample were desalted using 5 mL spin Sephadex G-25 columns previously equilibrated in 50 mM 2-(N-morpholino) ethanesulphonic acid (MES) buffer, pH 5.0. Elution of MUPs from these columns was as described above. Anion exchange chromatography was performed using a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech, Uppsala, Sweden) fitted with a Mono-Q column (bed volume, 1 mL) previously equilibrated with 10 mL of 50 mM MES buffer, pH 5.0. A 50 μL aliquot of desalted MUPs was applied to the column and bound protein was subsequently desorbed using a linear gradient of 0–200 mM NaCl in 32 mL. Fractions (0.5 mL) were collected between elution volumes of 2 and 32 mL. Protein content of the column eluent was monitored as absorbance at 280 nm in a 0.5 mL flow cell. Fractions containing discrete peaks of protein were pooled and then injected onto a reversed-phase desalting trap (bed volume, 7 μL) (Michrom BioResources, Pleasanton, CA, USA) which had previously been equilibrated with 300 μL of 0.2% (v/v) aqueous formic acid. MUPs bound to the trap were then washed with a further 300 μL 0.2% (v/v) formic acid prior to being eluted with a 100 μL aliquot of acetonitrile + water (80/20 v/v) containing 0.2% (v/v) formic acid. This solution was subsequently analysed, without dilution, by mass spectrometry.

Electrospray mass spectrometry

The mass spectrometer used was a Micromass Quattro (Altrincham, Cheshire, UK), upgraded to Quattro II specifications. Introduction of samples was by continuous infusion at 7 μL/min. Scanning was from m/z 600 to 1600 at a rate of 10 s/scan and at unit resolution. Eight to ten scans were averaged to give the final spectra. Calibration of the mass scale was achieved using a 2 pmol/μL solution of horse-heart myoglobin (M₉ 16951.5; Cat. no. M1882; Sigma Chemical Co., Poole, Dorset, UK) in acetonitrile + water (50/50 v/v) containing 0.2% (v/v) formic acid. Calibration was undertaken prior to data acquisition and after every two samples analysed thereafter. Sample spectra were m/z assigned using calibration data obtained from the adjacent myoglobin analysis.

Instrument control, data collection and processing were all achieved using the MassLynx package supplied with the instrument. Acquired m/z data were transformed onto a true mass scale and the resolution enhanced using Micromass maximum entropy software. All samples were processed at 1 mass unit/channel between the masses of 17500 and 19000 Da. A peak width at half height value of 0.75 m/z-units was determined from peaks in the myoglobin standards and applied to all samples.

RESULTS AND DISCUSSION

Our previous survey of MUPs from two inbred strains⁹,¹⁰ defined several components that were common to both. Several of these could be discerned from ESI-MS analyses of total urinary proteins after removal of low molecular weight urinary components on Sephadex G-25 spun columns. However, in the inbred mouse samples, there were several examples of MUPs with near-identical masses exhibiting different behaviour on ion-exchange media; the need for a chromatographic step prior to mass analysis was therefore indicated.

The maximum-entropy-processed ESI spectra of desalted urine samples from four wild mice are shown in Fig. 1. Previous analyses of these urine samples by SDS-PAGE and Western blotting (results not shown) demonstrated that they contained MUPs at similar concentrations to inbred strains. For each of these four wild-derived individuals, a complex pattern of masses and relative abundances was apparent. Moreover, the pattern of MUP expression varied between individual mice. Wild mice W-Dr, W-BGG5, and W-TM3 expressed proteins at 18 644 Da, 18 681 Da and 18 693 Da. W-Dr, W-R20, W-TM3 all expressed a protein at 19 709 Da but this was absent from W-BGG5. The protein of mass 18 644 Da was completely absent in

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W-R20, but this animal expressed a novel protein at 18644 Da. Replicate analyses of the above samples showed reproducibility to within ± 2 Da. The sample from W-BGG5 is particularly complex and includes a second group of components with apparent masses 98 Da higher than members of the major group. The experimental conditions employed for electrospray analysis suggest that the higher-mass constituents are unlikely to be associated with non-covalent adducts; further study is required, however, to elucidate their origin.

The overall picture is one of considerable complexity in MUP expression among wild mice. Fig. 2 summarizes, in bar-chart form, the analyses of unfractionated MUPs from eight wild mice, in addition to pooled urine from two inbred strains. Neither the proteins of mass 18681 Da (all except W-To1) and 18664 Da (W-R20 and W-TM3), nor the higher mass constituents noted in sample W-BGG5, have previously been observed in samples from inbred mouse strains. However, previous experience with MUPs from inbred strains suggested that some of the mass spectrometric peaks might comprise two proteins that were isobaric or too close in mass to be resolved under the experimental conditions employed. For example, we have previously demonstrated that a mass spectrometrically detected component of 18.693 Da was attributable to two different proteins in laboratory inbred mice, and that the two proteins were due to an isobaric substitution (Lys/Gln) which resulted in a different elution pattern on high resolution ion-exchange chromatography.10 We have detected additional MUP components after ion-exchange chromatography that were not previously evident from mass spectrometric analysis of the unfractionated sample. Accordingly, we have undertaken a combination of ion-exchange chromatography and mass analyses, and present here the data for W-R20.

The data obtained by combining ion exchange chromatography on a Mono-Q column with subsequent analysis of the separated peaks by ES-MS are shown in Fig. 3, for the example of MUPs from W-R20. The elution profile comprised a set of three major, closely spaced peaks and a fourth peak eluting at higher salt concentration. Each of the peaks was collected, desalted and concentrated prior to electrospray mass spectrometric analysis. As anticipated, some of the fractions were still heterogeneous in their MUP profile. Fractions corresponding to peaks 2 and 4 each contained a single mass, and confirmed the existence of the 18709 Da and 18878 Da proteins observed in the analysis shown in Fig. 1. Fraction 1 contained the proteins at 18664 Da and 18683 Da, neither of which had previously been observed in inbred strains. Fraction 3 comprised a protein of 18694 Da and a second protein of 18714 Da that was unresolved in the unfractionated sample. These late-eluting proteins that are rather anionic may correspond to components of similar mass expressed by inbred strains (e.g. the protein at 18693 Da in both BALB/c mice and C57BL/6J mice) (Fig. 2). An 18693 Da protein of similar charge

![Figure 2. Summary of relative abundances of components of the electrospray mass spectra obtained from analyses of unfractionated major urinary proteins from pooled samples from (a) two inbred strains (BALB/c and C57BL/6J) and from (b) eight wild mice (W-R20, W-Dr, W-BGG2, W-BGG5, W-TGG1, W-To1, W-To4 and W-TM3). Estimates of relative abundances were based on peak areas in the maximum-entropy-processed spectra.](image)

![Figure 3. (a) Analysis of major urinary proteins from a wild mouse (W-R20) using preparative anion exchange chromatography and electrospray mass spectrometry of individual fractions. (b) The data for three wild and inbred (BALB/c, C57BL/6J) mice are summarized for comparison.](image)

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(as judged by anion-exchange chromatography) has recently been identified by us through Lys-C peptide mapping as a new MUP and assigned the code uMUP-VIII.\textsuperscript{10} In total, W-R20 expresses six MUPs of which three have not been identified in inbred strains, either by cDNA sequence analysis or by direct observation.

It is our intention to use these MUPs as part of a fingerprinting technique to monitor deposition and usage patterns in semi-natural environments. We were therefore interested to analyse samples of uncertain past history to assess the likelihood of adduct formation or degradation. For these analyses (Fig. 4), we used two samples from another wild animal W-To4 and a pooled sample, W-May, taken from twelve mice trapped from a single population. In the W-To4 instance, the two samples were collected with and without faecal contamination. In the uncontaminated sample, the major MUP masses were similar to those seen in the other wild individuals we have analysed (Fig. 2). However, in the contaminated sample, although a similar pattern of peaks was observed, they were all displaced 375 Da lower in mass. This is the mass of the C-terminal tripeptide Ala-Arg-Glu sequence that is common to all MUPs thus far cloned and sequenced.\textsuperscript{10} The mass spectrometric data provide good evidence for a limited endoproteolytic attack on the C-terminal of the MUP. This is known from the three dimensional structure\textsuperscript{11} to be relatively flexible and might be expected to be vulnerable to degradation. The shift downwards by 375 Da, with no intermediate peaks, suggests the possibility of an endopeptidase attack rather than a sequential exopeptidase attack. We have recently characterized one such endopeptidase in mouse urine.\textsuperscript{13}

By contrast, the W-May pooled sample showed two clusters of peaks. The higher mass cluster corresponds to the values seen in the wild samples presented earlier (Fig. 2), but the lower cluster differs by a consistent 285 Da. This mass shift would be consistent with loss of the C-terminal dipeptide Arg-Glu. It is clear that this sample, with the added complexity of being of mixed origin, might have undergone two C-terminal exopeptidase attacks, or a single dipeptidyl carboxypeptidase degradation. Further, the tripeptide removal from the W-To4 sample must be interpreted in this context.

Additional work is needed to ascertain the sequence and timing of the terminal trimming that these proteins undergo. The trimming must occur post-release because the freshly-collected W-To4 sample showed no processing, in common with all the other samples analysed (Figs 1 and 2). The modification may therefore have value as an index of aged versus freshly deposited samples. At present, we do not know whether the C-terminal processing could influence patterns or rates of exfoliation and release from the MUPs.

The ESI-MS analysis of MUPs from wild mice has detected additional complexities not observed in inbred strains. ESI-MS of the unfractionated MUPs, with maximum-entropy enhancement of the spectra, has provided evidence of MUPs not previously observed. Additionally, the technique was capable of resolving a mixture of at least four MUPs and their partially proteolyzed products. The greatest amount of information, however, was obtained with the combined use of preparative anion exchange chromatography and ESI-MS, allowing individual MUPs to be characterized by two parameters, molecular mass and net charge in solution, inferred from anion exchange mobility. We expect to develop this system of allelic fingerprinting to analyse the distribution of MUP gene products within and between wild populations. Further analysis of the MUP patterns, in combination with behavioural analyses and analysis of samples deposited in wild environments, should lead to a clearer understanding of the role of these proteins in signalling. Finally, the discovery of new MUPs, specific to individual wild mice introduces opportunities for protein-specific tracking methods to explore patterns of MUP deposition and countermarking in interactions between individuals.

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