COMMUNICATION

OBC
www.rsc.org/obc

Non-denaturing electrospray ionisation-mass spectrometry reveals ligand selectivity in histamine-binding protein RaHBP2†

Neil J. Oldham,*[a] Olga Lissina,[b] Miles A. Nunn[b] and Guido C. Paesen[b]

[a] *The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford, UK OX1 3QY. E-mail: neil.oldham@chem.ox.ac.uk; Fax: +44 (0)1865 275674; Tel: +44 (0)1865 275657*

[b] *CEH Oxford, Mansfield Road, Oxford, UK OX1 3SR*

Received 17th June 2003, Accepted 23rd July 2003
First published as an Advance Article on the web 30th July 2003

ESI-MS has been used to probe the non-covalent interactions between a histamine-binding protein, from *Rhipicephalus appendiculatus*, and a range of bioactive amine ligands.

The ability of electrospray ionisation (ESI) both to impart multiple charges on a protein molecule and to desolvate the resulting ion, with minimal energy transfer, offers the possibility of studying weak biomolecular interactions by mass spectrometry (MS). Although several established methods for the analysis of intermolecular associations exist (*e.g.* circular dichroism, fluorescence spectroscopy, native gel electrophoresis and calorimetry), 'non-denaturing' ESI-MS allows direct visualisation of free and complexed species, as well as accurate determination of their masses (and hence stoichiometry). A wide variety of non-covalent protein assemblies have been observed by MS, including protein–protein and protein–small molecule complexes.[1] Of the latter class, several sub-groups can be identified, including enzyme–substrate,[2] enzyme–cofactor,[3] enzyme–inhibitor,[4] and binding/carrier-protein–ligand complexes.[5] The general requirements for detecting such non-covalent interactions, however, remain the same: namely to spray the protein from a near-neutral volatile aqueous buffer solution (*e.g.* NH_4OAc, 10 mM), with a low desolvation temperature (typically < 50 °C), and a reduced cone voltage setting (typically 20–50 V, but dependant on instrument type). In addition, the use of collisional cooling has been found to be effective in reducing the internal energy of non-covalent ion complexes, thereby preventing dissociation.[6]

Histamine-binding protein RaHBP2 is classified as a member of the lipocalin superfamily of lipid-binding proteins, based on its eight-stranded anti-parallel β-barrel architecture.[7] RaHBP2 differs from conventional lipocalins in several respects, most notably by possessing two binding cavities with affinity for polar, cationic ligands.[8] Secreted into its host by the ixodid tick, *Rhipicephalus appendiculatus*, the protein suppresses wound inflammation by binding locally-produced histamine (**1**), thereby allowing the tick to feed for relatively long periods of time without detection. Crystal-soaking experiments have indicated that the two binding sites have rather different affinities for the amine. The high-affinity site binds histamine with a K_d of 1.7 nM, whereas the low-affinity site has a K_d an order of magnitude higher, leading to the suggestion that histamine may not be the target ligand of this site. The potent antihistamine activity of RaHBP2, and related proteins, is of considerable medicinal interest and offers potential for treatment of allergic conditions such as conjunctivitis.[9] Non-denaturing ESI-MS provides an ideal approach for the study of RaHBP2-histamine binding, and for investigating the ligand selectivity of this protein. Here we report our findings on the non-covalent interactions between RaHBP2 and a range of bioactive amines using MS.

The surface of RaHBP2 is rich in acidic amino acid residues, resulting in a calculated p*I* of 3.9. It was decided, therefore, to analyse the protein by negative ion ESI-MS from a slightly basic buffer solution under carefully optimised native conditions.‡ The resulting mass spectrum exhibited ions at *m*/*z* 2265.8, 2549.1 and 2913.8, corresponding to the 9-, 8- and 7-charge states, respectively. Fig. 1 shows the $[M - 9H]^{9-}$ ion of RaHBP2 in the absence (a) and presence (b) of histamine (10-fold molar excess). The shift of *m*/*z* 12.3 and 24.7 in the lower spectrum corresponds to the presence of 1 and 2 histamine molecules in the RaHBP2 binding sites (M_r of histamine = 111.15, *m*/*z* = 12.35, *z* = 9). In the presence of excess amine, the ratio of RaHBP, [RaHBP2 + histamine] and [RaHBP2 + histamine$_2$] was found to be highly dependent upon cone voltage (Fig. 2). At 20–28 V, [RaHBP + histamine$_2$] was the dominant ionic species. However, the intensity of this ion decreased rapidly between 30 and 34 V, as the proportion of [RaHBP2 + histamine] and free RaHBP increased, with the single histamine complex showing a distinct peak at 31 V. Essentially no ligand binding is visible at a cone voltage of 38 V — as collisional activation completely disrupts the complex.

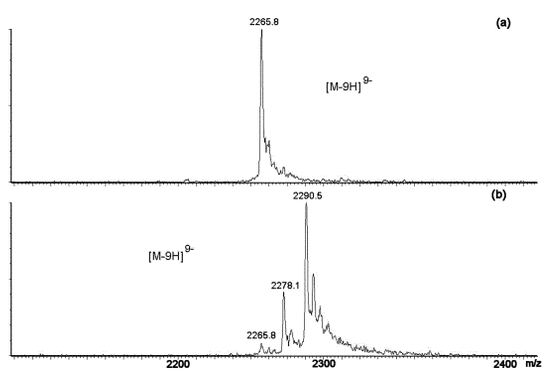


Fig. 1 'Native' ESI-mass spectra of RaHBP2 (a) alone and (b) in the presence of histamine (10-fold molar excess) showing the *m*/*z* shift of 1 and 2 multiples of 12.3 (*z* = 9), corresponding to the binding of 1 and 2 histamine molecules (M_r = 111.15). Cone voltage = 26 V.

Following successful observation of the RaHBP2–histamine complex, the ability of RaHBP2 to bind other bioactive amines was investigated. Solutions of protein were incubated with a 10-fold molar excess of dopamine (**2**), serotonin (**3**), tryptamine (**4**) or tyramine (**5**), or with a mixture of the five amines (each in a 4-fold excess). In the non-competitive assays, binding was observed with each amine (Fig. 3, Fig. 1b). Based on the intensities of RaHBP, [RaHBP2 + amine] and [RaHBP2 + amine$_2$] ions, however, a clear order of affinity could be identified, with

† This is one of a number of contributions from the current members of the Dyson Perrins Laboratory to mark the end of almost 90 years of organic chemistry research in that building, as all its current academic staff move across South Parks Road to a new purpose-built laboratory.

DOI: 10.1039/b306929j

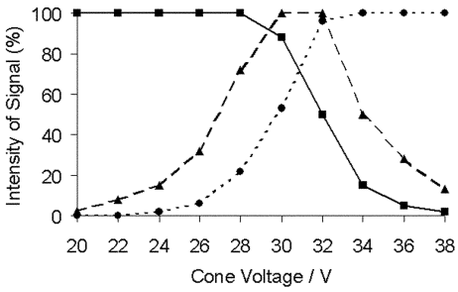


Fig. 2 Cone voltage-induced fragmentation of [RaHBP2 + histamine$_2$] non-covalent complex. Legend: ■ = [RaHBP2 + histamine$_2$]; ▲ = [RaHBP2 + histamine]; ● = RaHBP2. All data derived from the $[M - 9H]^{9-}$ charge state.

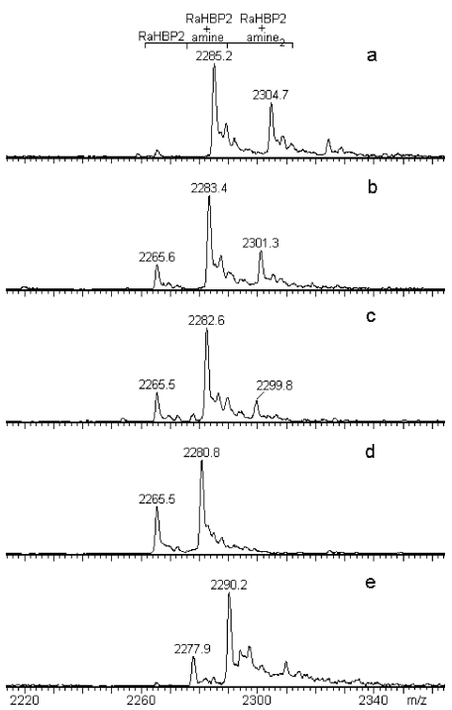
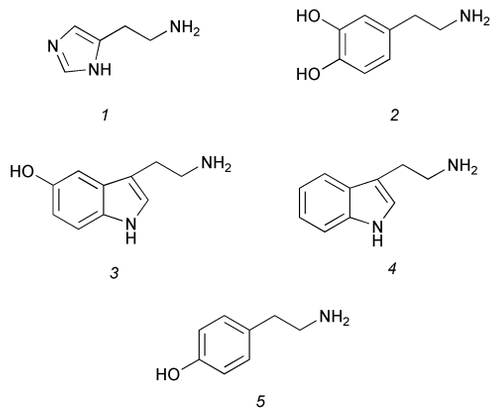


Fig. 3 'Native' ESI-MS of RaHBP2 (charge state $[M - 9H]^{9-}$) after exposure to (a) serotonin, (b) tryptamine, (c) dopamine, (d) tyramine, and (e) all of the above + histamine. Cone voltage = 26 V.

histamine > serotonin > tryptamine > dopamine > tyramine. The spectrum obtained from exposing all five amines to RaHBP2 was almost identical to that for histamine (Figs. 1b and 3e), demonstrating that in a competitive assay RaHBP2 selectively binds histamine in both binding sites. It is noteworthy that the indolic amines serotonin and tryptamine bind more effectively than the phenolic dopamine and tyramine, presumably by better mimicking the imidazole ring of histamine. Furthermore, presence of a hydroxy group on C3 of the benzene ring, and on the corresponding C5 of the indole ring system appears to improve binding (*cf*. dopamine > tyramine; serotonin > tryptamine).

In an attempt to provide quantitative ligand-binding data for RaHBP2, aliquots of protein solution (20 μM) were incubated with histamine at a range of concentrations between 2 and 40 μM. Analysis by non-denaturing ESI-MS revealed that, to within experimental error, the concentration of bound histamine was equal to the total concentration of histamine added. Thus, the amount of free histamine in solution was too low for accurate determination, preventing calculation of K_d. This result highlights a general problem with quantification of binding by MS when K_d is in the low nM range (or lower). Since the MS approach detects both free and complexed protein, it is necessary to add relatively high concentrations of ligand (>10 mol%) before the [protein–ligand] ions appear in the



spectrum. Current work is directed towards a competitive displacement assay using histamine and serotonin, which should overcome this problem. The presence of two binding sites, with potentially dependent K_d values complicates this approach, however.

In conclusion, this work shows that it is possible to detect a complex between the histamine-binding protein RaHBP2 and a variety of amine ligands using electrospray-mass spectrometry. Moreover, the results confirm that this unusual type of lipocalin is capable of binding two amine molecules. While the extremely high affinity of RaHBP2 for histamine prevented determination of K_d, the competitive binding assay revealed selectivity for histamine over a range of aromatic bioactive amines. Both binding sites of RaHBP2 showed a clear preference for histamine, demonstrating that serotonin, tryptamine, dopamine and tyramine are not primary target ligands of this protein. The next stage of this work is to screen libraries of histaminergics and melatonin receptor ligands using ESI-MS to provide structure–activity data for this and related histamine binding proteins, in order that we may better understand the ligand selectivity of this important group of novel antihistamines.

Notes and references

‡ Mass spectrometry was performed on a Micromass LCT (time of flight mass spectrometer) equipped with a Z-Spray™ ESI source and operated in the negative ion mode. RaHBP2 (His-tagged), typically 20 μM, was sprayed from NH_4OAc (10 mM, pH 7.5) at a flow rate of 10 μl min^{-1}, with nitrogen as a nebuliser and desolvation gas at 300 l hr^{-1} (total flow) and 40 °C. Instrument settings were optimised using a standard solution of CsI to give maximum signal strength at a resolution of 3000 (FWHM). A capillary voltage of 2060 V was used throughout, and the cone voltage was adjusted between 20 and 40 V to control dissociation of non-covalent complexes. RaHBP2 was incubated with potential ligands (various concentrations) for 1 h at 25 °C before analysis by ESI-MS.

1 J. A. Loo, *Mass Spectrom. Rev.*, 1997, **16**, 1; T. D. Veenstra, *Biophys. Chem.*, 1999, **79**, 63.
2 B. Ganem, Y.-T. Li and J. D. Henion, *J. Am. Chem. Soc.*, 1991, **113**, 7818.
3 A. Van Dorsselaer, N. Potier, P. Barth, D. Trisch and J.-F. Biellmann, in *Proceedings of the 44th ASMS Conference on Mass Spectrometry and Allied Topics*, 1996, Portland, OR, p. 1407.
4 M. Baca and S. B. H. Kent, *J. Am. Chem. Soc.*, 1992, **114**, 3992; R. T. Aplin and C. V. Robinson, in *Mass Spectrometry in the Biological Sciences*, eds. A. L. Burlingame and S. A. Carr, Humana Press, Totowa, NJ, 1996, p. 69.
5 N. J. Oldham, J. Kreiger, H. Breer, A. Fischedick, M. Hoskovec and A. Svatos, *Angew. Chem., Int. Ed*, 2000, **39**, 4341.
6 M. A. Tito, J. Miller, K. F. Griffin, E. D. Williamson, R. W. Titball and C. V. Robinson, *Protein Sci.*, 2001, **10**, 2408.
7 G. C. Paesen, P. L. Adams, K. Harlos, P. A. Nuttall and D. I. Stuart, *Mol. Cell*, 1999, **3**, 661.
8 G. C. Paesen, P. L. Adams, P. A. Nuttall and D. I. Stuart, *Biochim. Biophys. Acta*, 2000, **1482**, 91.
9 M. J. Chapin, P. J. Gomes, P. Nuttall, G. Paesen and M. B. Abelson, *Invest. Opthalmol. Visual Sci.*, 2000, **41**(S-365), 1916.