Does the Observation of Noncovalent Complexes between Biomolecules by Electrospray Ionisation Mass Spectrometry Necessarily Reflect Specific Solution Interactions?

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Electrospray ionisation mass spectrometry is used to observe complexes formed between porcine pancreatic elastase (PPE) and its peptidic substrates and products; analogous complexes are observed between PPE and the enantiomers of the substrates and products.

Electrospray ionisation mass spectrometry (ESI MS) has been used both to characterise covalently inhibited enzymes[1] and to observe complexes consistent with the formation of covalent intermediates in enzyme-catalysed reactions.[2] The observation of structurally specific *noncovalent* complexes by ESI or ion spray (IS) MS has also been reported.[3] The observed complexes are generally interpreted as resulting from the preservation of *structurally specific* noncovalent solution phase interactions into the gas phase by the soft ionisation technique. However, the observation of noncovalent cluster ions or multimers which do not reflect structurally specific interactions in dilute solution by ESI and IS MS has also been demonstrated.[3,4]

We have reported ESI MS studies on the inhibition of porcine pancreatic elastase (PPE) by several inhibitors of elastase.[1,5] We now describe studies using ESI MS on PPE-catalysed hydrolysis of peptidic substrates, which should stimulate further studies directed towards addressing the question of the structural specificity of noncovalent complexes observed by ESI and IS MS.

Incubation of PPE† with the *p*-nitroanilide substrate, MeOSuc-L-Ala-L-Ala-L-Pro-L-Val-4-$NHC_6H_4NO_2$ **1**[6] at an initial PPE concentration of 90 pmol μl^{-1} and ESI MS[5] analysis resulted in the observation of mass shifts corresponding to the formation of a complex between **1** and PPE [C and D, Fig. 1(*a*); entry 1, Table 1]. Increasing the PPE concentration at which the incubation was carried out to 200 pmol μl^{-1}, whilst maintaining other experimental parameters, resulted in the production of mass shifts corresponding to complexes between PPE and **2**, the hydrolysis product of **1** [G and H, Fig. 1(*b*); entry 2, Table 1]. The concomitant production of *p*-nitroaniline was also observed by ESI MS analysis. Analogous mass shifts were observed when **2** was incubated with PPE [G and H, Fig. 2(*b*); entry 3, Table 1]. Experiments in which PPE was firstly irreversibly inhibited with MeOSuc-L-Ala-L-Ala-L-Pro-L-ValCH_2Cl **3**[5] before incubation with substrate **1** led to the observation of relatively small (<10%) amounts of new mass shifts (which were effectively removed by increasing the cone voltage) corresponding to the addition of an intact molecule of **1** to the inhibited enzyme. Formation of *p*-nitroaniline was not observed in this case.

MeOSuc-L-Ala-L-Ala-L-Pro-L-Val-R^1
1 R^1 = 4-$NHC_6H_4NO_2$
2 R^1 = OH
3 R^1 = CH_2Cl
4 R^1 = OEt,
MeOSuc-D-Ala-D-Ala-D-Pro-D-Val-R^2
5 R^2 = OEt
6 R^2 = OH

On incubation of ester **4** with PPE it was possible to observe by ESI MS analysis mass shifts corresponding to complexes formed between PPE and **4** and/or PPE and acid **2** (entry 4, Table 1).

To explore the specificity of the binding of peptides to PPE and hence the nature of the interactions preserved or formed during the ESI process, the enantiomer **5** of the ester **4** was incubated with PPE and the ESI MS analysis performed. Mass shifts corresponding to the formation of a 1:1 complex between **5** and PPE [M and N, Fig. 2(*a*); entry 5, Table 1] were observed. Concomitant production of acid **6** [*i.e.* the enantiomer of the hydrolysis product **2** of *p*-nitroanilide **1** or ester **4**] was not observed by ESI MS, implying that no hydrolysis of **5** had occurred. The acid **6** was shown to form a complex with PPE [Q and R, Fig. 2(*c*); entry 6, Table 1] at a similar ratio (relative to virgin PPE) to that observed with its enantiomer **2** [G and H, Fig. 2(*b*)].

Incubation of an equimolar mixture of **1** and **5** with PPE (90 pmol μl^{-1})† resulted in the observation of mass shifts corresponding to PPE: **1** (mass shift = 591.4 ± 4, calc. = 590.64 Da) and PPE: **5** (mass shift = 497.7 ± 8, calc. = 498.58 Da) complexes in *ca.* 3:1 ratio respectively, apparently indicating significant binding of **5** to PPE, even in the presence of **1**. These experiments would seem to imply that *either PPE*

Table 1 Averaged observed mass shifts compared to native PPE following incubations with **2**, **2**, **4**, **5** and **6**[a]

Entry	Peptide (X), PPE: (X) ratio[a]	Incubation times used/min	Mass Shift on PPE-asn/Da[b]	Mass Shifts on PPE/Da[b]	Calc. Mass Shift for PPE: (X) 1:1 complex/Da
1 [Fig. 1(*a*)]	**1**, 1:1	<1, 1, 3, 5	587.4 (17)	590.3 (22)	PPE:**1** 590.64
2 [Fig. 1(*b*)]	**1**, 1:1	1, 3	468.8 (2)	467.7 (3)	PPE:**1** 470.53
3 [Fig. 2(*a*)]	**2**, 1:3	1, 3	468.4 (2)	472.4 (2)	PPE:**2** 470.53
4	**4**, 1:3	<1, 1	471.0 (3)	471.7 (7)	PPE:**2** 470.53
			500.3 (1)	499.8 (4)	PPE:**4** 498.58
5 [Fig. 2(*b*)]	**5**, 1:3	1, 3	498.51 (4)	499.27 (4)	PPE:**5** 498.58
6 [Fig. 2(*c*)]	**6** 1:3	<1	463.9 (1)	471.59 (2)	PPE:**6** 470.53

[a] PPE concentration = 90 pmol μl^{-1}, except for entry 2, where it was 200 pmol μl^{-1}. [b] Numbers in parentheses = number of experiments over which data has been averaged.

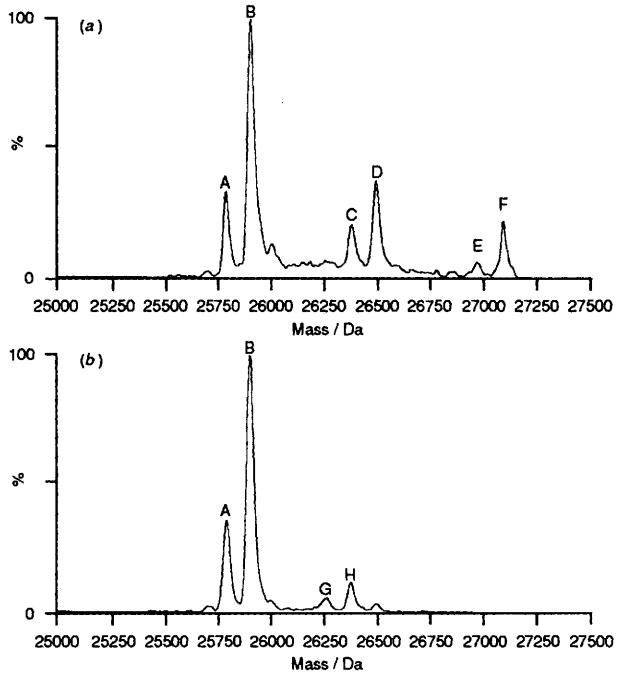

Fig. 1 Mass transformed data following ESI MS analysis of (*a*) PPE (90 pmol μl^{-1}) incubated with **1**, cone voltage = 50 V, (*b*) PPE (200 pmol μl^{-1}) incubated with **1**, cone voltage = 50 V. For experimental details and observed mass shifts, see Table 1.‡

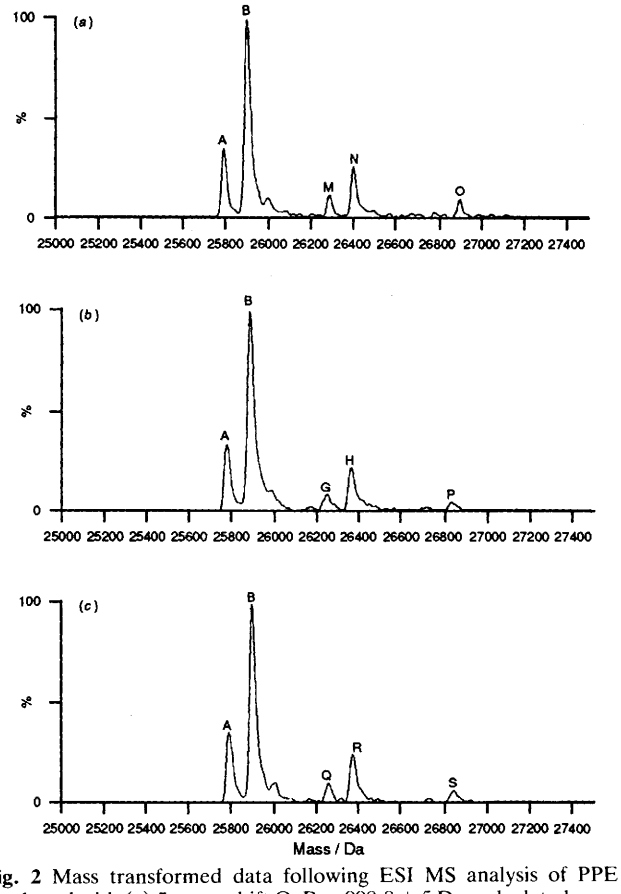

Fig. 2 Mass transformed data following ESI MS analysis of PPE incubated with (*a*) **5**, mass shift O–B = 998.8 ± 5 Da, calculated mass shift for formation of PPE : **5** 1 : 2 complex = 997.16 Da; (*b*) **2**, mass shift P–B = 940.6 ± 6 Da, calculated mass shift for formation of PPE : **2** 1 : 2 complex = 941.06 Da; (*c*) **6**, mass shift S–B = 940.6 ± 5 Da, calculated mass shift for formation of PPE : **6** 1 : 2 complex = 941.06 Da. For experimental details and other observed mass shifts, see Table 1.

is very structurally nonspecific in terms of binding, or the observation of these complexes by ESI MS does not fully *reflect structurally specific binding in dilute solution*. No reduction in the initial rate of hydrolysis of **1** by PPE was observed when a solution of **1** (at fixed substrate concentration)[6] was treated with a solution of PPE containing **6** (5 mmol dm^{-3}), supporting the latter interpretation. To our knowledge, there is no evidence for the enantiomer of a naturally occurring oligo- or poly-peptide substrate displaying significant binding to the active site of a protease.[7] Indeed, Kent *et al.*[8] have shown that the two enantiomeric forms of HIV-1 protease exhibit reciprocal chiral specificity to substrates and inhibitors.

It may be argued that the mass shifts observed on incubation of PPE with **1**, **2** and **4** may correspond to the first and second tetrahedral intermediates proposed in the mode of hydrolysis of substrates by elastases,[9] but it is difficult to reasonably interpret the observed mass shifts for **5** and **6** in this manner.

Throughout this study, small amounts of additional mass shifts consistent with the addition of two substrate/product molecules to PPE were observed.‡ Since it is known that elastases have an extended binding pocket[10] and that the active site of PPE can incorporate two molecules of Ac-L-Ala-L-Pro-L-Ala,[11] it may be argued that the observation of mass shifts corresponding to the binding of a second molecule of substrate or product to PPE by ESI MS may result from a specific interaction in solution. However, the formation of mass shifts corresponding to the binding of the enantiomeric substrate **5** or product **6** argue against such an assumption. In addition the largest peptide to have been shown to bind simultaneously in two different places at the active site of elastase is a tripeptide with a relatively small N-terminal protecting group [acetyl[12] *c.f.* the methoxysuccinyl present in **1**, **2**, **4**, **5** and **6**]. Furthermore, using a magnetic sector instrument§ it was possible to observe mass shifts corresponding to the formation of complexes in which three or more tetrapeptides were bound to PPE.

Few studies[12] have been carried out on the formation, preservation or destruction of interactions between molecules in the ESI or IS processes, but such research should be of enormous interest in addressing the noncovalent specificity question. The results presented here indicate that the observed complexes between PPE and peptides do not always (fully) reflect structurally specific interactions in solution. It is clear that there is a specificity requirement for the observation of PPE : peptide complexes since several randomly chosen peptides did not give rise to mass shifts. It could be that this specificity requirement arises (at least partially) from the nature of interactions between PPE and the peptide which occur during the ESI process, where conditions may be very different from those used for analyses in dilute solution, *i.e.* the relative strength of interactions between PPE and peptides that are formed during the electrospray process may be very different from the strength of these interactions in dilute solution. Thus, at this stage we believe it is not possible to give a definitive *general*[13] answer to the question as to whether or not the observation of 'noncovalent' complexes by ESI MS results from the preservation of structurally specific interactions in solution,[3] but given the enormous potential of this technique it is clearly an important issue which will have to be addressed by those working in the field.

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Footnotes

† As previously reported,[1,5] the commercially available PPE used in our studies consists of two components of observed M_r = 25784.8 ± 1.5 and M_r = 25898 ± 1.5 [A and B, Fig. 1(*a*)], corresponding to PPE without its C-terminal asparagine residue (theoretical M_r = 25784.06) and 'intact' PPE (theoretical M_r = 25898.06), respectively. In the studies described subsequently, analogous mass shifts were generally observed corresponding to binding to both of these species. In some experiments where the intensities of the observed mass shifts were low, only the shifts corresponding to binding to intact PPE were used in the calculation of the average mass shifts presented in Table 1. In the competition experiments described, a second batch of PPE which did not contain any C-terminal truncated PPE was used in order to simplify the ESI MS spectrum obtained.

‡ For example, on incubation of PPE with **1** mass shifts corresponding to the formation of a PPE : **1** 1 : 2 complex were observed [E and F, Fig. 1(*a*); PPE (90 pmol μl^{-1}) : **1** 1 : 1 or 1 : 3, incubation time = 1,3 min; mass shifts: E–A = 1179.4 Da (averaged over 3 experiments); F–B = 1177.4 Da; calculated mass shift for formation of PPE : **1** 1 : 2 complex = 1181.28 Da]. These additional mass shifts were not observed when the cone voltage was increased to 70 V. Concomitant depletion in the intensity (relative to the virgin PPE) of the mass shifts corresponding to the addition of one molecule of **1** was also observed when ESI MS analysis was carried out at a cone voltage of 70 V relative to experiments carried out at a cone voltage of 50 V. Literature precedent[3] would suggest that these cone voltage experiments imply the observed complexes are noncovalent, although unstable covalent complexes (*e.g.* tetrahedral intermediates) cannot be ruled out. Other examples shown in this paper are: Fig. 2(*a*), O; Fig. 2(*b*), P; Fig. 2(*c*), S; see Fig. 2 legend for details.

§ A Fisons Instruments VG Autospec 5000 was used.

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