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Some electrospray mass spectrometric evidence for the existence of covalent *O*-acyl enzyme intermediates

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Electrospray mass spectrometry has been used to measure the masses of the species present in solutions of three serine proteases (α-chymotrypsin, subtilisin Carlsberg and subtilisin BPN') before, during and after completion of the hydrolytic reaction with cinnamoyl imidazole and indole acryloyl imidazole. The masses measured during the reaction demonstrated that covalent *O*-acyl enzyme intermediates had been formed.

Electrospray mass spectrometry; Serine protease; *O*-Acyl enzyme

1. INTRODUCTION

The twin problems of the mechanism of action and source(s) of the catalytic power of enzymes have been the subject of extensive study for many decades. Whilst the solution to the latter problem still eludes us, plausible explanations as to the mode of action of many enzymes and of the serine proteases in particular have been established and are generally accepted. According to current theory [1] the overall catalytic process of these proteolytic enzymes may be divided into two distinct stages, the first being an acylation which is then followed by a deacylation reaction. It is postulated that the acylation stage results in the transfer of the acyl moiety of the substrate to the hydroxyl group of the active serine residue with the formation of a covalent *O*-acyl enzyme. The subsequent deacylation reaction, involving an 'activated' water molecule, regenerates the enzyme.

The evidence for this theory is based upon the interpretation of the accumulated wealth of kinetic data and on deductions drawn from X-ray crystallographic and chemical studies of the enzymes and their natural and synthetic model substrates. Additional direct evidence in support of the formation of the key covalently bonded *O*-acyl enzyme intermediate has been afforded by detailed kinetic and structural studies of certain small molecule chromophoric substrates. Thus the UV and visible absorption spectra of solutions of the intermediate products obtained by reacting α-chymotrypsin, trypsin, subtilisin Carlsberg and subtilisin BPN' (Novo) have been interpreted previously by one of us [2–4] as providing good evidence for the existence of covalent *O*-acyl enzyme intermediates. Further support for this interpretation of the solution electronic spectra came from the determination of the crystal structure of indole acryloyl-α-chymotrypsin [5] which firmly established the covalent nature of the binding of the acyl group to the oxygen atom of the active serine in the solid state.

Since 1988 a new, rapid and sensitive electrospray mass spectrometric method (ES-MS) [6] for the determination of the molecular masses of large intact proteins has been in existence and this is now being successfully employed by an increasing number of researchers [7]. Arising from a detailed study of abnormal haemoglobins [8], it became clear to us that this new technique would provide an excellent method for confirming the presence of covalently bonded groups in proteins, since as practised to date, with aqueous organic solvents, the method has invariably resulted in the dissociation of non-covalently bonded species such as haem groups. In this paper we present the results of a series of electrospray mass spectrometric experiments, which parallel the earlier electronic spectroscopic studies [2–4] in an attempt to provide further direct evidence for the existence of the covalently bound *O*-acyl enzyme intermediates in solution.

2. EXPERIMENTAL

2.1. *Materials*

Cinnamoyl imidazole (CI), C12,H10,N2,O; M_r = 198, and α-chymotrypsin (bovine) were purchased from Sigma Chemical Company, indole acryloyl imidazole (IAI), C14,H11,N3,O; M_r = 237, was a gift

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Table I

Measured molecular masses (Da) of species present in solutions of serine proteases and the reaction mixtures formed with cinnamoyl imidazole (CI) and indole acryloyl imidazole (IAI) at various times t (min), together with the calculated molecular masses of all theoretically possible intermediates

Protease	M_r sequence*	M_r exptl. t_o	Substrate	M_r calc. enzyme substrate complex	M_r calc. enzyme product (acid) complex	M_r calc. enzyme product (imidazole) complex	M_r calc. *O*-acyl enzyme	M_r exptl. t_x min.		
Subtilisin Carlsberg	27 288	27 289	CI	27 486	27 436	27 356	27 418	t_5 27 419	t_{32} 27 291	–
			IAI	27 525	27 475	27 356	27 457	t_5 27 460	t_{35} 27 460	t_{491} 27 293
Subtilisin BPN'	27 534	27 536	CI	27 732	27 682	27 602	27 664	t_5 27 670	t_{50} 27 667	t_{1113} 27 537
			IAI	27 771	27 721	27 602	27 703	t_5 27 706	t_{32} 27 706	t_{1173} 27 534
α-Chymotrypsin (minor component)	25 234	25 237	CI	25 432	25 382	25 302	25 364	t_5 25 363	t_{43} 25 365	t_{1043} 25 264
			IAI	25 471	25 421	25 302	25 403	t_5 25 404	t_{44} 25 404	t_{1066} 25 264
α-Chymotrypsin (major component)	–	25 450	CI	25 648†	25 598†	25 518†	25 580†	t_5 25 580	t_{43} 25 581	t_{1043} 25 430
			IAI	25 687†	25 637†	25 518†	25 619†	t_5 25 619	t_{44} 25 620	t_{1066} 25 432

*Enzyme sequence data taken from Dayhoff, Atlas of Protein Sequence and Structure.
†Masses calculated with respect to M_r exptl t_o

from Dr C.W. Wharton of the Biochemistry Department, University of Birmingham. Subtilisin Carlsberg and subtilisin BPN' were a gift from Dr I. Svendsen of the Chemical Department, Carlsberg Research Centre, Copenhagen.

2.2. *Methods*

Electrospray mass spectra were measured with a VG BioQ triple quadrupole mass spectrometer equipped with an electrospray interface and data handling system. Samples (10 μl) of the test solution (25–50 pmol/μl) were injected directly into the electrospray source via a loop injector (Rheodyne 5717) at a solvent (water/acetonitrile, 50:50, v/v, formic acid 1%) flow rate of 4 μl·min^{-1} (Pharmacia pump 2248). Sample solutions were prepared immediately prior to analysis by mixing 1:7 molar proportions of the enzyme and the chromophoric substrates in (Milli-Q) water at pH 5.2, this pH being optimum for the formation of a stable *O*-acyl intermediate [4]. The excess substrate ensures that the enzyme will be predominantly in the acylated form. Aliquots (25 μl) of the reaction mixtures were taken at appropriate time intervals, mixed with an equal volume of MeCN containing 1% (v/v) formic acid and immediately analyzed by electrospray mass spectrometry. The mass spectrometer was routinely scanned over the m/z range 800–1600 and this mass scale was calibrated using a solution of horse heart myoglobin (20-pmol injections) M_r = 16 951.48 Da. After 7 h, any of the reaction mixtures which still showed the presence of the *O*-acyl enzyme were made basic (pH≈9) with ammonia and left overnight. The mixtures were then mass measured, after adjusting to pH≈5.0 by the addition of a suitable aliquot of formic acid, as described above.

3. RESULTS

Initially the mass of each enzyme studied was determined (M_r exptl, t_o Column 3 Table I) before reaction with the substrate to confirm the authenticity of the materials by comparison with the calculated values from the published amino acid sequence (M_r sequence; Column 2). Several of the enzymes showed the presence of other minor components but in the case of α-chymotrypsin the enzyme itself was a minor component in the sample, with the major component giving an M_r of 25 450 Da. All the measured M_r's agreed with those calculated from the published sequences, where known, to within 0.012%.

A corresponding analysis of the reaction mixtures was then made at appropriate time intervals (M_r exptl, t_x; Column 9). Fig. 1 affords an illustrative example of the electrospray mass spectrum obtained from subtilisin Carlsberg (a) before, (b) during and (c) after reaction with indole acryloyl imidazole. As can be seen from the figure, during the reaction of M_r exptl increases from 27 289 to 27 460 Da, indicating that the *O*-acyl enzyme has been formed (M_r calculated 27 457 Da; Column 8 Table I). In all cases the measured masses at t=5 min corresponded to within 0.022% of those calculated for the covalently bonded *O*-acyl enzyme intermediates (see Table I).

A further study of the data presented in Table I (Column 9) shows that with the exception of the cinnamoyl subtilisin Carlsberg, all the *O*-acyl enzymes were evident for periods in excess of 30 min. The data also shows that at extended times the *O*-acyl subtilisins

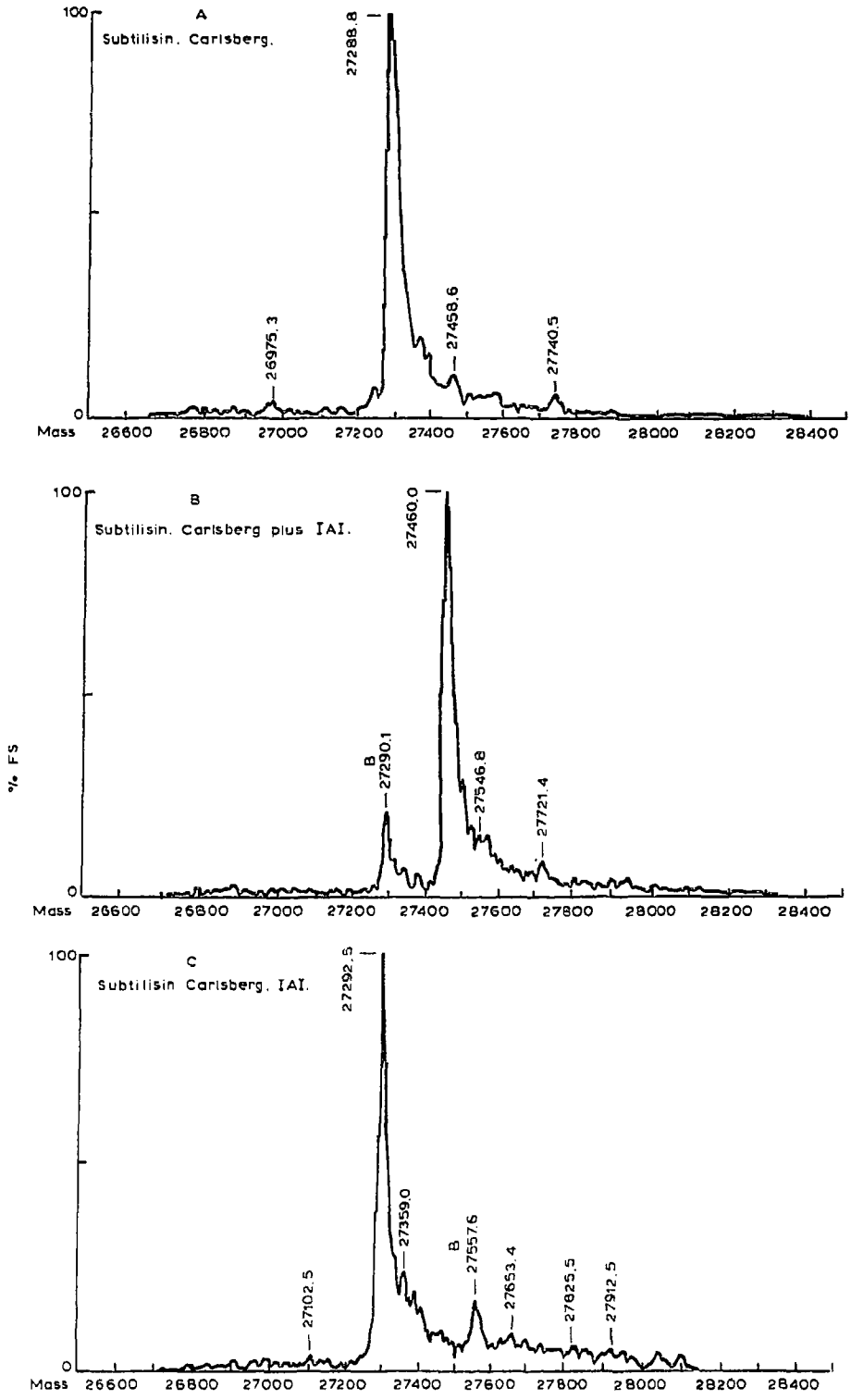

Fig. 1. (a) Electrospray mass spectrum from Subtilisin Carlsberg. (b) Electrospray mass spectrum from Subtilisin Carlsberg after reaction with indole acryloyl imidazole for 5 min. (c) Electrospray mass spectrum from Subtilisin Carlsberg after reaction with indole acryloyl imidazole for 491 min.

all suffered deacylation with consequent regeneration of the original enzymes. However, in the case of α-chymotrypsin solutions the measured M_r's at extended times do not correspond to those expected for the deacylated enzyme and further studies are now in progress to elucidate the origin of these unexpected results.

The data presented here also suggests that the major component observed in the electrospray mass spectrum

of the α-chymotrypsin is probably a related serine protease since the measured masses of the reaction mixture at intermediate times show major peaks corresponding to the mass of the *O*-acyl enzyme for this component (Column 9 Table I). This *O*-acyl enzyme shows similar anomalous behaviour to that observed for α-chymotrypsin itself, namely that the enzyme does not appear to be regenerated upon completion of the hydrolytic reaction, as evidenced by the measured masses; and again this is currently the subject of further study.

4. DISCUSSION

The detection of *O*-acyl enzyme intermediates by ES-MS has been previously reported for the β-lactamases [9], however this pioneering study did not fully demonstrate that the intermediate was covalently bonded because the predicted masses of the non-covalent enzyme–substrate complexes and the covalently bonded *O*-acyl enzymes were identical. In the present study, the masses of the covalently bonded *O*-acyl enzymes and the various theoretically possible non-covalently bonded intermediate complexes differ by at least 18 Da (see columns 5–8 Table I), so that it may be safely inferred from the measured masses that these correspond to the covalently bonded *O*-acyl enzyme intermediates and not to any of the listed non-covalently bonded complexes.

The speed (typically less than 5 min) and the utility of the ES-MS technique as demonstrated here indicates that enzyme kinetics may be readily studied by this method. Further, the evidence presented here that the major component of the α-chymotrypsin sample behaves as a serine protease also indicates the potential of the ES-MS technique for detecting the presence of new serine proteases and new *O*-acyl enzyme intermediates even in impure preparations.

Finally, a recent report has indicated that, by modifying the tip of the electrospray needle, ES-MS may be performed on aqueous solutions thereby enabling the presence of non-covalently bonded intermediates to be demonstrated. Hopefully this advance may provide the means whereby all the intermediates in an enzyme reaction may be studied mass spectrometrically.

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