Inhibition of TEM-2 β-Lactamase from *Escherichia coli* by Clavulanic Acid: Observation of Intermediates by Electrospray Ionization Mass Spectrometry[†,‡]

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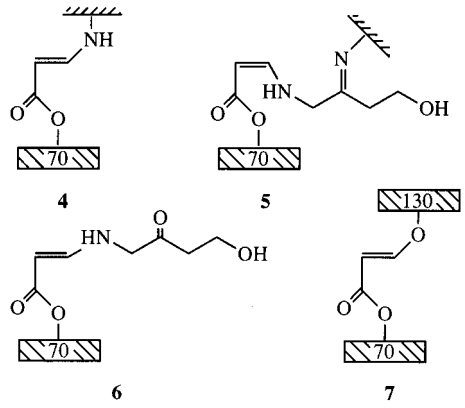
Received May 1, 1996; Revised Manuscript Received July 11, 1996[⊗]

ABSTRACT: Clavulanic acid, the therapeutically important inhibitor of β-lactamases containing a nucleophilic serine residue at their active sites, inhibits *Escherichia coli* TEM-2 β-lactamase via a complex mechanism. Electrospray ionization mass spectrometry (ESIMS) studies revealed that a minimum of four different modified proteins are formed upon incubation of clavulanate with the TEM-2 enzyme. These exhibit mass increments relative to the unmodified TEM-2 β-lactamase of 52, 70, 88, and 155 Da. Time course studies implied that no long-lived forms of clavulanate-inhibited TEM-2 β-lactamase retain the carbons of the oxazolidine ring of clavulanate. The absence of a 199 Da increment to unmodified TEM-2 suggests rapid decarboxylation of clavulanate upon binding to the enzyme. Proteolytic digestions of purified forms of clavulanate inhibited TEM-2 β-lactamase followed by analyses using high-performance liquid chromatography coupled to ESIMS (HPLC-ESIMS) and chemical sequencing were used to provide positional information on the modifications to the enzyme. Increments of 70 and 80 Da increments were shown to be located in a peptide containing Ser-70. A further 70 Da mass increment, assigned as a β-linked acrylate, was localized to a peptide containing Ser-130. A mechanistic scheme for the reaction of clavulanate with TEM-2 β-lactamase is proposed in which acylation at Ser-70 and subsequent decarboxylation is followed either by cross-linking with Ser-130 to form a vinyl ether or by reformation of unmodified enzyme via a Ser-70 linked (hydrated) aldehyde. Purified cross-linked vinyl ether was observed to slowly convert under acidic conditions to a Ser-70 linked (hydrated) aldehyde with concomitant conversion of Ser-130 to a dehydroalanyl residue.

β-Lactamase catalyzed hydrolysis of the therapeutic β-lactams is the major cause of bacterial resistance to such antibiotics. β-Lactamases may be classified (Bush, 1989) on a structural basis (Ambler, 1980), separating the metalloenzymes (class B) from those that involve a nucleophilic active site serine residue in their hydrolysis mechanism. The serine enzymes are further divided into classes within which there is considerable sequence homology: class A (penicillinases), class C (cephalosporinases) (Jaurin & Grundstrom, 1981), and now class D (Huovinen et al., 1987). Diversity in the genetic organization underlying the synthesis and regulation of β-lactamases, unrelated to functional or structural classification, also exists. Genes may be chromosomal or plasmid located in both gram positive and negative bacteria. The location of the β-lactamase gene on plasmids allows transfer between cells; their location on transposons allows transfer between replicons. The potential therefore exists for the particularly facile spread of β-lactamase mediated resistance between bacteria (Richmond et al., 1980). The evolution of resistance has necessitated the development of counterstrategies; the use of the serine β-lactamase inhibitor clavulanic acid (**1**), in conjunction with the susceptible β-lactam antibiotic, has been one successful approach.

Spectroscopic studies on clavulanate inhibition of the class A β-lactamase from *Staphylococcus aureus* led to the proposed inhibition mechanism shown in Figure 1 (Cartwright & Coulson, 1979, 1980; Rizwi et al., 1989). Decarboxylation of the β-keto acid **3a** was suggested by Reading and Hepburn (1978), who also speculated on the possibility of involvement of a lysine residue in forming the imine **5**.



Detailed studies on the class A *Escherichia coli* RTEM enzyme demonstrated the existence of three irreversibly inactivated species in addition to a transiently inhibited form that slowly regenerated active enzyme (Charnas et al., 1978). ^{14}C-Labeling experiments (Charnas & Knowles, 1981) implied that, of the four inactivated species, the carbons of both rings of the clavulanate skeleton were retained in the transiently inhibited and two of the three irreversibly inactivated species. On the basis of a strong absorption at 280 nm, the transiently inhibited species was assigned as

[†] This work was supported by the E.P.S.R.C., the M.R.C., the B.B.S.R.C., and SmithKline Beecham Pharmaceuticals Ltd.

[‡] Dedicated to the memory of Stephen Waley, who died 6th December 1993.

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[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.

S0006-2960(96)01044-6 CCC: $12.00

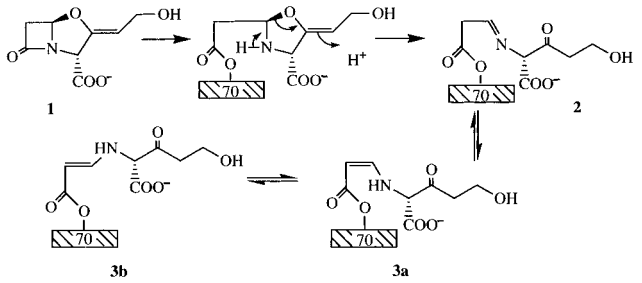


FIGURE 1: Proposed mechanism for clavulanate inhibition of β-lactamase from *S. aureus* (Cartwright & Coulson, 1980).

the enamine **3a**. Cross-linking with a lysine residue to form the enamine **4** was proposed to explain the single irreversibly inactivated species that retained the three carbons of the β-lactam ring alone (Brenner & Knowles, 1984; Knowles, 1985).

High-resolution crystal structures for several class A β-lactamases have afforded a detailed insight into active site structures and hence an appreciation of the possible roles of various residues during inhibition by clavulanic acid (**1**). Subsequent to determination of the crystal structure of β-lactamase from *S. aureus* PC1 (Herzberg, 1991), a 2.2 Å resolution cryocrystallographic study on a trapped enzyme−clavulanate complex proved consistent with the formation of the Ser-70-attached[1] *cis*-enamine **3a** as well as decarboxylated *trans*-enamine **6** (Chen & Herzberg, 1991). Resolution was not, however, sufficient to distinguish between the imine **2** and the *cis*-enamine **3a**. No evidence for cross-linking was observed, and indeed Lys-73 was considered the only residue realistically positioned for such a role. In contrast, the 2 Å crystal structure of the *Bacillus licheniformis* β-lactamase (Moews et al., 1990) allowed modeling studies leading to the proposal that, while transimination involving either Lys-73 or Lys-234 again seemed sterically unlikely, Ser-130 was well situated for an analagous cross-linking role to form the vinyl ether **7** which would also be consistent with the previous spectroscopic studies (Imtiaz et al., 1993). Site-directed mutagenesis and modeling studies on the homologous plasmid-mediated class A ROB-1 enzyme provided further evidence for a structural and substrate-binding role for Ser-130 (Jacob et al., 1990; Juteau et al., 1992).

Electrospray ionization mass spectrometry (ESIMS)[2] has been used to characterize covalently inhibited enzymes, including serine proteases (Aplin et al., 1992, 1993a; Ashton et al., 1991) and serine β-lacatmases (Aplin et al., 1990a, 1993b; Saves et al., 1995). ESIMS studies on the interaction of poor substrates and inhibitors with the serine β-lactamases led to the observation of species corresponding to acylated enzymes (Aplin et al., 1990b) only in those cases where prior kinetic studies had indicated this would be feasible (Galleni & Frere, 1988). Thus, in these cases it appears that the observation of adducts by soft ionization mass spectrometry reflects specific solution interactions. In other cases, particularly for the characterization of noncovalent adducts by ESIMS, the situation is less clear (Aplin et al., 1994; Smith & Light-Wahl, 1993; Busman et al., 1994). When coupled to HPLC (HPLC-ESIMS), the utility of ESIMS is extended to provide positional information regarding disulfide bridging and covalent protein modification (Robertson et al., 1994; Griffin et al., 1991; Roach et al., 1994). Here, we describe the use of ESIMS and HPLC-ESIMS to investigate the complex inhibition of the *E. coli* TEM-2 β-lactamase by clavulanic acid (**1**). This work provides not only information on the mass of several enzyme−inhibitor complexes but also, for the first time, direct evidence for the involvement of Ser-130, in addition to Ser-70, in the inhibition process.

EXPERIMENTAL PROCEDURES

Materials. TEM-2 β-lactamase from *E. coli* was obtained from Public Health Laboratory Service Centre for Applied Microbiology and Research, Porton Down, Salisbury, U.K. Potassium clavulanate and *tert*-butyl ammonium clavulanate were gifts of SmithKline Beecham Pharmaceuticals Ltd., Brockham Park, Betchworth, Surrey, U.K. Endoproteinase Glu-C (*S. aureus* V8 protease) and sequencing grade modified trypsin were purchased from Promega. All reagents used were spectroscopic grade or higher.

Electrospray Ionization Mass Spectrometry. Intact protein ESIMS was performed using a VG Bio-Q triple quadrupole atmospheric pressure mass spectrometer equipped with an electrospray ionization interface. Solvent (acetonitrile/water, 1:1 v/v) was supplied at a flow rate of 8 μL min^{-1} using an Applied Biosystems 140A solvent delivery system. After appropriate incubation in 20 mM Tris-HCl buffer, pH 7.8, reactions were quenched by addition of an equal volume of acetonitrile containing 2% (v/v) formic acid such that the final mixture was composed of water/acetonitrile/formic acid in proportions of 1:1:1%. The final protein concentration was typically 20 pmol μL^{-1}. Myoglobin, when used as an intensity calibrant, was present at a concentration of 8 pmol μL^{-1}. Mass spectra typically comprised of 15 10 s scans over the range 750−1500 Da or, for higher resolution spectra, 15 8 s scans over the range 1020−1170 Da. Under Results, masses are reported to the nearest 0.5 Da. Mass spectra were acquired using a cone voltage of 50 V and a source temperature of 50 °C. Data processing was performed using Mass Lynx 1.0, VG Analytical, Altrincham, U.K.

HPLC-Electrospray Ionization Mass Spectrometry. HPLC-ESIMS was performed using a VG Platform single quadrupole atmospheric pressure mass spectrometer equipped with an electrospray ionization interface. HPLC was performed using an Applied Biosystems 140B solvent delivery system equipped with an 112A oven-injector, 785A programmable absorbance detector, and JJ instruments CR 550 chart recorder. Solvent A was composed of water containing 2% (v/v) acetonitrile and 0.1% (v/v) TFA. Solvent B was composed of acetonitrile containing 20% (v/v) water and 0.1% (v/v) TFA. Separation was achieved using a 250 mm column, with an internal diameter of 1 mm, packed with Synchropak C-18, particle size of 6.5 μm eluting with a 5−80% (v/v) solvent B gradient over 55 min at a flow rate of 50 μL min^{-1}. Where intact proteins were analyzed, the packing was Hypersil C-4, particle size 5 μm, and they were eluted as above except that a 55−75% (v/v) solvent B gradient over 24 min was used. Microbore PEEK tubing (Upchurch Scientific) with an internal diameter of 0.0025 in. was used throughout. The eluant was divided post column between mass spectrometer and absorbance detector,

[1] The numbering system for class A β-lactamase residues uses the ABL scheme (Ambler et al., 1991).

[2] Abbreviations: HPLC, high-pressure liquid chromatography; ESIMS, electrospray ionization mass spectrometry.

typically in a 1:2 ratio, using a PEEK tee-piece. Digested samples, derived from an original β-lactamase concentration of approximately 1 mg mL^{-1}, were diluted with twice their volume of solvent A before analysis. Mass spectra were acquired by repeatedly scanning from 380 to 1500 Da in centroid mode over 2 s. Data analysis was performed using Mass Lynx 2.0 (VG Analytical) software.

HPLC Purification of Clavulanate-Inhibited TEM-2 β-Lactamase. Purification of clavulanate-inhibited β-lactamase by reverse-phase HPLC was performed using a Waters 600E system controller equipped with a 490E multiwavelength detector and SE 120 chart recorder. In a typical procedure TEM-2 β-lactamase (100 μL of a 10 mg mL^{-1} solution in 20 mM Tris-HCl, pH 7.8) was incubated with potassium clavulanate (100 μL of a 120 mM solution in the same buffer) for 1 h. The mixture was desalted using Bio-Rad Bio-Spin gel filtration columns (Bio-Gel P-6 gel matrix) before injection onto a 250 mm × 4.6 mm Vydac protein C4 column. Elution was performed using a 45−70% (v/v) solvent B gradient at a flow rate of 1 mL min^{-1} over 28 min following a 2 min isocratic period at the initial conditions. This procedure separated the modified enzyme into two fractions: fraction 1 (retention time 19 min) and fraction 2 (retention time 23.5 min). Collected fractions were lyophilized and stored at −80 °C until use. Inhibition by 6β-iodopenicillanic acid (6β-IPA) was achieved by adding 100 mL of a 1 mM solution of 6β-IPA in 20 mM Tris-HCl buffer, pH 7.8, to an equal volume of a 1 mg mL^{-1} solution of β-lactamase in the same buffer. The mixture was incubated for 10 min, and proteolytic digestion was performed without further manipulation.

Proteolysis. To effect proteolytic digestion of β-lactamase, 20 μg of protease was dissolved in 100 μL of 20 mM Tris-HCl buffer at pH 7.8. In those digestions performed under reducing conditions the buffer also contained 2.5 mM DTT. For control digestions and those involving partially purified clavulanate-inhibited β-lactamase, protease solution was added directly to lyophilized protein (approximately 100 μg), and, after vigorous shaking, digestions were performed at 37 °C for the reported time. For digestions involving β-lactamase inhibited by 6β-iodopenicillanic acid, 120 μL of a 0.5 mg mL^{-1} solution of inhibited enzyme, in 20 mM Tris-HCl, pH 7.8, was added to 20 μg of the lyophilized protease.

RESULTS

Intact Protein ESIMS

Initial attempts using ESIMS to obtain masses for inhibited forms of TEM-2, using potassium clavulanate, afforded mass spectra which, despite demonstrating some mass shifts relative to uninhibited enzyme, were unacceptably broadened due to the presence of potassium adducts. The situation was improved by using *tert*-butyl ammonium clavulanate as inhibitor. Typical experiments using this inhibitor apparently revealed three series: Series A corresponding to unmodified TEM-2 (calculated molecular mass 28 905.24 Da), series X consisting of broad peaks seemingly corresponding to a broad band of approximate mass 28 980 Da and the less intense series B corresponding to a mass shift of 154 ± 7 Da relative to native enzyme. The line width of series X was greater than those of series A and B, and the mass it implied on repetition of the acquisition was variable. These observations, in conjunction with the results of maximum entropy data analyses (Ferrige et al., 1992; Skilling, 1992), suggested that series X might have resulted from the superposition of a number of unresolved series. Higher resolution spectra were then acquired scanning between 1020 and 1170 Da to include the four charge states $z = 26-29$ only. Figure 2a shows such a reduced range mass spectrum, acquired after a 1 min incubation at a *tert*-butyl ammonium clavulanate/enzyme ratio of 18:1. That the broad series X observed in low resolution ESIMS mass spectra was in fact a superposition of at least three series (C, D, and E) is, especially at lower *z* values, now apparent. Figure 2b shows the associated output following maximum entropy data analysis, from which the values in Table 1 were obtained. Table 1 shows that the component series C, D, E, and B of Figure 2a, correspond to species approximately 52, 70, 88, and 155 Da greater in mass respectively than native TEM-2 β-lactamase (series A).

In order to quantify the amount of the five species present at a given time myoglobin was used as an internal intensity calibrant. Following incubation with a *tert*-butylammonium clavulanate:enzyme ratio of 18:1, Figure 3a illustrates the return to dominance of native β-lactamase and slow decline in intensity of series C. Figure 3b illustrates the broadly parallel, more rapid declines in intensity of series D and E and the even shorter lifetime of component B. Note that the interpretation of this analysis assumes that the intensity of each series is proportional to the concentration of the relevant species.

HPLC-ESIMS

Digestion of Unmodified TEM-2 β-Lactamase. It was hoped that proteolytic digestion followed by HPLC-ESIMS would reveal the site(s) of covalent modification of TEM-2 β-lactamase by clavulanate. Initial experiments were performed monitoring the digestion with endoproteinase Glu-C of unmodified TEM-2. The resultant assignments accounted for the entire TEM-2 sequence except for the three N-terminal residues (26−28). These were observed in peptides 26−37 and 26−48 after shorter digestion times or when digestion was performed at pH 4 (Table 2). Note in particular that a peak arising from disulfide-bridged double peptide (65−89)+(122−145) that contains the active site residues Ser-70, Lys-73, and Ser-130 was observed in this case.

Digestion (Endoproteinase Glu-C) of Crude Clavulanate-Inhibited TEM-2 β-Lactamase. Initial attempts to observe modified peptides, using crude clavulanate-inhibited enzyme, provided chromatograms very similar to those obtained from control experiments; they apparently contained neither modified peptides nor the absence of unmodified peptides from around the active site. It was speculated that the observed similarity between the digestion products of these experiments and those of control experiments was due in part to the presence of native enzyme in the predigestion mixture rather than hydrolysis of inactivated enzyme under the digestion and/or analysis conditions. Such interference by native β-lactamase was presumably worsened by the observed greater susceptibility to proteolytic digestion of unmodified, as compared to modified, enzyme.

Digestion (Endoproteinase Glu-C) of Partially Purified Clavulanate-Inhibited TEM-2 β-Lactamase. Using reverse-

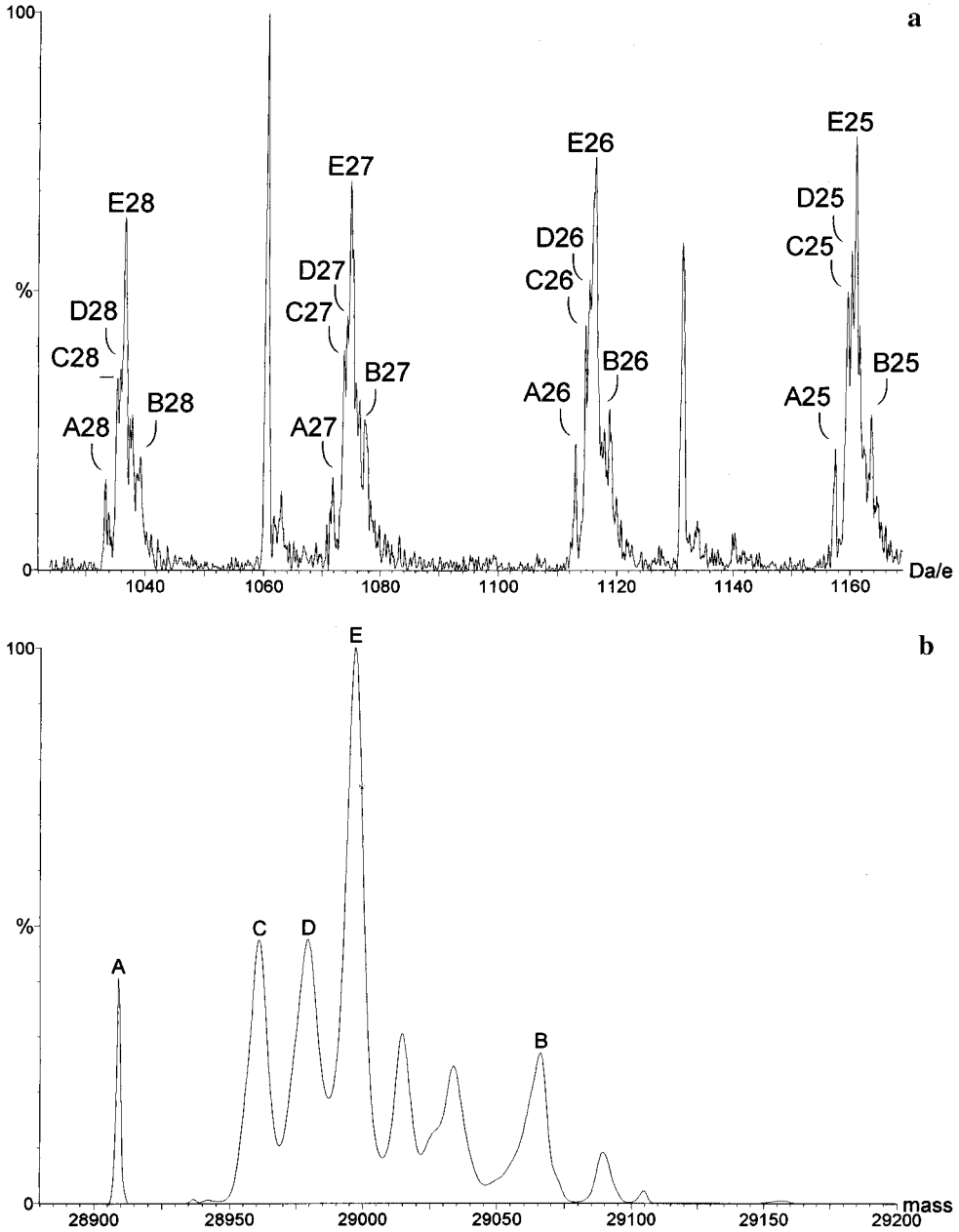


FIGURE 2: (a) Reduced mass range spectrum ($z = 26, 27, 28, 29$) acquired after a 1 min incubation of *tert*-butylammonium clavulante with TEM-2 β-lactamase. (b) Data resulting in panel a after maximum entropy processing.

Table 1: Masses Obtained from Spectrum in Figure 2[a]

component	mass (Da) (maximum entropy)[a]	mass shift from TEM-2 (Da)[b]
A	28 909.1 ± 0.5	
B	29 064.6 ± 2.5	155.5 ± 3.0
C	28 960.7 ± 2.0	51.6 ± 2.5
D	28 979.1 ± 2.6	70.0 ± 3.1
E	28 996.8 ± 1.1	87.7 ± 1.6

[a] Masses implied from a maximum entropy generated mock data set [ME(Ev-14010, It 13)(Gs 0.6, 1024−1169, 0.50, L50 R50); Sb (1, 33.00)]. [b] Mass shifts derived by subtraction of observed mass of unmodified enzyme (A).

phase HPLC, it was possible both to remove unmodified enzyme from the predigestion mixture and to purify differently modified TEM-2 β-lactamase species. The modified enzyme eluted in two fractions: fraction 1 and fraction 2. Subsequent ESIMS analysis showed *fraction 1 to contain enzyme apparently modified with mass increments of 52 and 70 Da relative to unmodified TEM-2*. Fraction 1 was observed to slowly degrade on standing (in aqueous acetonitrile containing 0.1% trifluoroacetic acid) to principally yield a protein with a mass of 28 888 Da. This mass is consistent with the production of *dehydrated* TEM-2 (calculated mass 28 887 Da) from the modified protein in fraction 1. There was no evidence for the production of unmodified TEM-2 from fraction 1, but the presence of a low level (<10%) cannot be ruled out. *Thus, the mass increments of the modified TEM-2 present in fraction 1 are 70 and 88 Da relative to dehydrated TEM-2.*

ESIMS analysis of *fraction 2 again demonstrated both 52 and 70 Da mass increments relative to unmodified TEM-2.* It should be noted there was no evidence for the presence of a 88 Da mass increment, although the presence of a low level (<20%) cannot be ruled out. In contrast to the behavior observed for fraction 1, however, there was no evidence for the direct conversion of the modified protein present in

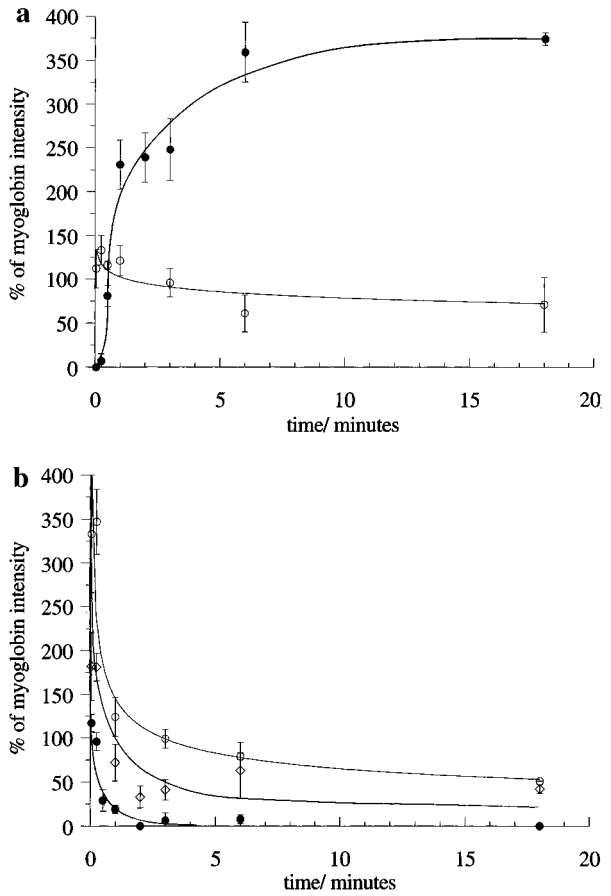


FIGURE 3: (a) Time course for mass changes to TEM-2 β-lactamase after incubation with *tert*-butylammonium clavulanate. Solid circles, native TEM-2 enzyme; empty circles, TEM-2 + 52 Da. Reported intensities are areas of maximum entropy generated peaks expressed as percentages of the myoglobin calibrant spectrum intensity. Illustrated errors are standard deviations derived from multiple determinations. (b) Time course for mass changes to TEM-2 β-lactamase after incubation with *tert*-butylammonium clavulanate. Solid circles, native TEM-2 + 155 Da enzyme; empty circles, TEM-2 + 88 Da: diamonds, TEM-2 + 70 Da. Reported intensities are ares of maximum entropy generated peaks expressed as percentages of the myoglobin calibrant spectrum intensity. Illustrated errors are standard deviations derived from multiple determinations.

Table 2: Assignments for HPLC-ESIMS Analysis of Partially Purified Clavulanate-Inhibited TEM-2 (Fraction 2) following Digestion with Endoproteinase Glu-C (1 h)[a]

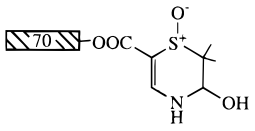
peak no.	retention time (min)	mass (Da)[a]	assignment[b]	expected mass (Da)
1	9.7	886.3	275−281	885.98
2	12.1	694.8[c]	105−110	694.74
3	13.3	763.7[c]	59−64	763.81
4	15.0	1002.8	29−37	1002.18
5	16.3	1286.5	111−121	1286.48
6	20.4	1829.1 ± 1.5	90−104	1828.01
7	22.5	1102.1	49−58	1101.27
8	24.4	2205.3	29−48	2204.55
	24.4	1024.4	282−290	1024.23
9	25.9	1840.4	148−163	1840.09
10	27.2	839.3[c]	215−240‡	2511.91
11	28.0	3418.	241−274	3417.89
	28.0	2538	148−168	2537.84
12	31.0	2698.6	213−240	2698.08
13	31.6	1817.09[c]	198−212	1817.14
14	36.0	2177.39	178−197	2177.58
15	37.0	2816.4	172−197‡	2817.24
16	38.3	7896.04 ± 3.5	198−274‡	7897.08
	38.3	8764.37 ± 3.5	198−281‡	8565.04
17	44.0	10057.79 ± 4	178−274	10056.64
	44.0	10695.29 ± 2	172−274	10696.30
	44.0	10925.98 ± 2	178−281	10924.60
	44.0	11566.63 ± 2.5	172−281	11564.23
18	45.2	12569.87 ± 6	172−290‡	12570.48
	45.2	12931.31 ± 2	169−290‡	12926.86
19	46.4−49.5	~**29000**	***modified TEM-2 unresolved forms***‡	
20	49.8	**28957.91** ± **7.11**	TEM-2 + 52.67	28957.25

[a] Except where indicated in the third column the standard deviations were less than 1 Da from the mean reported mass. Reported masses were obtained from the sum of all scans below the appropriate peak. [b] Assignments in this and subsequent tables and are quoted using the ABL numbering scheme (Ambler et al., 1991). [c] Mass was derived from a single charge state. All assigned peptides were observed upon HPLC-ESIMS analysis of native TEM-2 following digestion (4 h) with endoproteinase Glu-C [except for those indicated by a double dagger (‡)]. Additional peaks assigned in the case of the unmodified TEM-2 enzyme corresponded to peptides 116−121, 29−35, 38−48, 167−197, 180−197, and 65−89+122−147 (i.e., disulfide linked) and the Glu-C proteinase were also observed. Digestion of native TEM-2 at pH 4 (5.5 h) yielded additional peaks assigned as corresponding to peptides 26−37 and 26−48.

fraction 2 to give dehydrated or unmodified TEM-2. Instead fraction 2 was observed to slowly convert (in aqueous acetonitrile containing 0.1% trifluoroacetic acid) into a mixture contining material exhibiting the ESIMS spectra and retention times characteristic of both fractions 1 and 2.

Use of inhibited enzyme partially purified by the reverse-phase HPLC procedure did not, in itself, facilitate observation of modified peptides, but now, using endoproteinase Glu-C and fraction 2, the disulfide-bridged double peptide (65−89)+(122−145) was *entirely invisible* (Table 2). Enzyme that had remained undigested was clearly modified, however. All other peptides from the control experiment were present. It was suspected that the absence of *any* information about this the active site region of the sequence was due to its dilution into a mixture of weak components. Such a dilution might result from heterogeneity arising from the imperfect partial purification and/or heterogeneity due to imperfect digestion. Heterogeneity due to imperfect digestion is multiplied when a disulfide bridge is present—there now being four termini to consider. Cross-linking by clavulanate would cause an analogous effect. Had hydrolysis of modified peptides been the principal problem then the observation of unmodified peptides containing Ser-70 would have been expected; their absence suggested that this was not the case. Repetition of the experiment, aiming to optimize sensitivity, failed to lift the intensity of putative modified peptides above background noise.

Digestion (Endoproteinase Glu-C) of 6β-Iodopenicillanic Acid-Inhibited TEM-2 β-Lactamase. On HPLC-ESIMS analysis of 6β-iodopenicillanic acid-inhibited TEM-2 following digestion with endoproteinase Glu-C the unmodified acylation site disulfide-bridged double peptide (65−89)+(122−145) was again entirely absent, but now an additional peptide with mass 5558 Da, consistent with the addition of 198 Da to the double peptide, was visible. Modified undigested enzyme with a mass of 29 103 Da, consistent with a modification of 198 Da, was also observed. The expected mass shift for this inhibitor is 198.31 Da. The unexpected presence of a species of mass 5545 ± 2 was also demonstrated. This mass may be explained by a modification of 186.6 Da to the disulfide-bridged double peptide, consistent with formation of the autoxidation product **8** as predicted



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by Orlek et al. (1980). The calculated mass shift for this modification is 187.22 Da. The relative ease of experiments using 6β-iodopenicillanic acid, an inhibitor for which only one major inhibited species is reported and inhibition is completely irreversible within the time scale of the experiment, suggested that heterogeneity resulting from the complex mechanism of clavulanate inhibition, despite the partial purification procedure, played a part in the invisibility of acylation site peptides.

Digestion (Endoproteinase Glu-C) of 6β-Iodopenicillanic Acid-Inhibited TEM-2 β-Lactamase in the Presence of DTT. That imperfect digestion also contributed to the dilution of active site peptides was confirmed by experiments using 6β-iodopenicillanic acid in the presence of DTT. HPLC-ESIMS analysis of 6β-iodopenicillanic acid-inhibited TEM-2 following digestion with endoproteinase Glu-C in the presence of DTT showed that under these reducing conditions not only were several modified peptides consistent with the addition of 6β-IPA to the active site Ser-70, observed in addition to modified undigested enzyme, but these peaks were significantly more intense than were those arising from the 6β-IPA modified disulfide-bridged double peptides obtained in the absence of DTT. Masses of 2954 (consistent with a mass increment of 198 on peptide 65−89), 4764.5 (consistent with a mass increment of 198 on peptide 65−104), and 5509 Da (consistent with a mass increment of 197 on peptide 59−104) were observed. A mass spectrum corresponding to unmodified peptide 122−145 (2606 Da) was obtained, demonstrating that only the Cys-77 half of the disulfide-bridged double peptide is modified, as expected from covalent modification at Ser-70 by the inhibitor.

Digestion (Endoproteinase Glu-C) of Fraction 2 in the Presence of DTT. The intensity enhancement caused by performing the digestion in the presence of DTT proved sufficient to allow the observation of active site peptides derived from clavulanate-inhibited enzyme (Table 3). Figure 4a shows part of the retention time vs mass map obtained from HPLC-ESIMS analysis of partially purified clavulanate-inhibited TEM-2 (fraction 2) following digestion with endoproteinase Glu-C in the presence of DTT. The presence of modified peptides was detected in peaks 14, 19, and 20. Peak 19 contains three co-eluting species of mass 5312, 5381.5, and 5440 Da (Figure 4c). These masses correspond to unmodified peptide 59−104 and peptide 59−104 modified by 69.5 and 88 Da, respectively. N-Terminal sequencing of collected peak 19 afforded the sequence SFRPEER which is consistent with these assignments. Also visible (peak 20, Table 3) was a peptide of mass 5195 (Figure 4d). This mass is consistent with peptide 122−168 modified by 69 Da. Similarly a peptide of mass 2675 Da (peak 14, Table 3), corresponding to peptide 122−147 modified by 69 Da, was observed. Furthermore, unmodified peptides containing Cys-123, having been clearly visible in control experiments and for example when using 6β-iodopenicillanic acid in reducing conditions, were entirely absent. After slightly longer digestion times (4 h) smaller additional modified peptides were visible: Figure 4e shows the mass spectrum of three species eluting in the leading edge of peak 19 showing masses 3501.5, 3571, and 3589 Da. These masses correspond to unmodified peptide 59−89 and peptide 59−89 modified by 69.5 and 87.5 Da, respectively. Although only isolated as a mixture with the modified 59−104 peptides above, with which they share the N-terminus, N-terminal sequencing of this mixture revealed only peptides beginning at Ser-59 (SFRPEER). All other regions of the protein were accounted for, in their unmodified state.

Table 3: Assignments for HPLC-ESIMS Analysis of Partially Purified Clavulanate-Inhibited TEM-2 (Fraction 2) following Digestion with Endoproteinase Glu-C in the Presence of DTT (2 h)[a]

peak no.	retention time (min)	mass[b] (Da)	assignment[c]	calculated mass (Da)
13	33.2	6966.4	203−281	6965.92
	33.2	6098.2	203−274	6097.95
14	34.3	2675.1	122−147 + 69 Da[d]	2606.01
16	37.4	2816.9	172−197	2817.24
17	38.5	8764.5	198−281	8565.04
18	39.0	7895.0 ± 5	198−274	7897.08
19	39.6	5312.0	59−104	5312.10
		5381.6	59−104 + 69 Da[e]	5312.10
		5400	59−104 + 88 Da[e]	5312.10
20	41.5	5195.0	122−168 + 69 Da[d]	5125.84
22	51.0	***2860.0*** ± ***6***	TEM-2 + 55 Da[d]	28905.24

[a] Peptides exhibiting a mass increment relative unmodified TEM-2 peptides are highlighted in bold italics. Except where indicated in column three, all standard deviaitons were less than 2 Da from the reported mass. [b] Reported masses were obtained from the sum of all scans below the appropriate peak. [c] Assignments are quoted using the ABL numbering scheme (Ambler et al., 1991). [d] Mass shift derived by subtracting calculated mass of unmodified peptide. [e] Mass shift derived by subtracting observed mass of unmodified peptide. In addition to the peaks aasigned in Table 3, peaks assigned as corresponding to peptides 275−281, 105−110, 111−121, 90−104, 49−58, 29−48, 282−290, 148−168, 241−274, 203-240, 198−212, 178−197, 172−274, 178−281, and 172−281 were observed.

Digestion (Tryptic) of Fraction 2 in the Presence of DTT. Assignments for the HPLC-ESIMS analysis of partially purified clavulanate-inhibited TEM-2 (fraction 2) following digestion with trypsin in the presence of DTT are shown in Table 4. Modified peptides were observed in peaks 27 and 28. Peak 27 has a mass of 2675.5 Da corresponding to peptide 121−146 modified by 69.5 Da. The sequences Arg-Glu at residues 120−121 and Lys-Glu at residues 146−147 mean that, coincidentally, this mass is identical to that of modified 122−147 in the endoproteinase Glu-C experiments described above (see Table 2). The mass spectrum derived from peak 28 showed principally unmodified peptide 66−83 (series A, 2001 Da), but a small amount of peptide 66−83 modified by 69.5 Da was also clearly present (ratio of unmodified/modified, ca. 3.5:1). The increased proportion of such unmodified Ser-70 containing peptide after tryptic digestion, in addition to the generally more complex and congested nature of resulting HPLC-ESIMS analyses, was one reason for concentrating largely on the use of endoproteinase Glu-C in this work. The rest of the spectrum is assigned in Table 4 in which all other regions of unmodified sequence are accounted for.

Digestion of Fraction 1 in the Presence of DTT. Figure 5 shows the total ion current chromatogram from HPLC-ESIMS analysis of partially purified clavulanate-inhibited TEM-2 (fraction 1) following digestion with endoproteinase

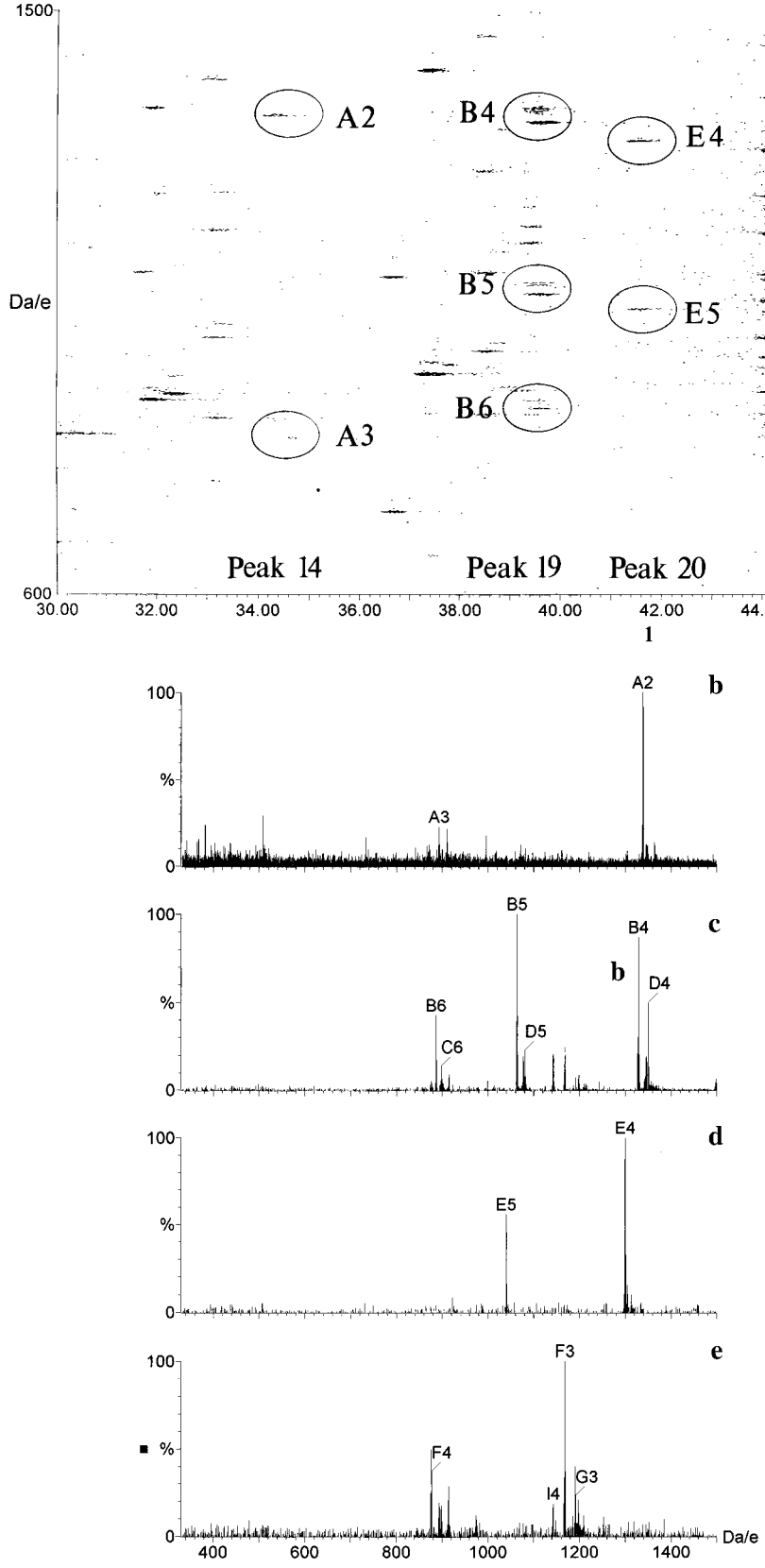


FIGURE 4: (a) Part of the retention time versus mass map for HPLC-ESIMS analysis of partially purified clavulanate-inhibited TEM-2 β-lactamase (fraction 2, see text) following digestion with endoproteinase Glu-C in the presence of DTT (2 h). [Res (1.00 Da, 1 scan); 2.24−20%; log scaling]. Important features from peaks 14, 19, and 20 are highlighted. (b) Mass spectrum (A) derived from peak 14, panel a, assigned as peptide 122−149 modified by 69 Da (A). (c) Mass spectrum derived from peak 19, panel a, showing the presence of three species, B (5312 Da), C (5381 Da), and D (5400 Da), assigned as unmodified peptide 59−104 and peptide 59−104 modified by 69.5 and 88 Da, respectively. (d) Mass spectrum (E) derived from peak 20, panel a, assigned as peptide 122−168 modified by 69 Da. (e) Mass spectrum derived from the leading edge of peak 19 panel a, after a further 2 h digestion. The three species, F (3501 Da), G (3570 Da), and I (3589 Da), were assigned as unmodified peptide 59−89 and peptide 59−89 modified by 69.5 and 87.5 Da, respectively.

Table 4: Assignments for HPLC-ESIMS Analysis of Partially Purified Clavulanate-Inhibited TEM-2 (Fraction 2) following Digestion with Trypsin in the Presence of DTT (3 h)[a]

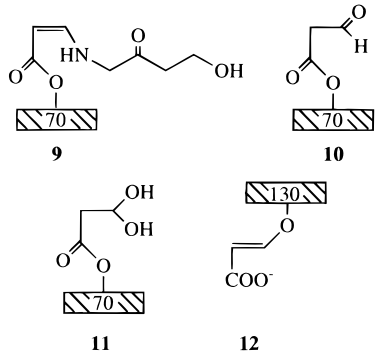
peak no.	retention time (min)	mass[b] (Da)	assignment[c]	calculated mass (Da)
1	4.4	804.6	33−39	803.87
2	4.9	416.2[d]	40−43	415.49
4	8.5	993.0	*h*	
5	10.6	524.3[d]	*h*	
6	10.9	823.7	26−32	822.96
7	11.5	1072.3	84−93	1072.14
8	12.3	590.9[d]	*h*	
9	14.8	1029.7	74−83 or 112−120[e]	1030.30 or 1029.18
10	18.5	1276.2	56−65[f]	1275.43
11	19.1	1608.4[d]	26−39	1608.81
12	20.1	1352.9	245−259	1351.57
13	20.6	1784.1	260−275	1784.02
14	20.9	1380.0	179−192	1379.62
15	21.3	2022.1	95−111	2022.20
16	21.9	1711.8[d]	165−178	1711.81
17	22.6	1307.4	44−55	1307.47
	22.6	1142.8	278−288	1142.36
	22.6	988.1	66−73	988.24
18	23.0	2097.3	162−178	2096.24
19	23.3	1030.7	74−83 or 112−120[e]	1030.30 or 1029.18
20	24.2	1376.5	205−215	1376.55
21	25.1	1741.8	147−161	1740.96
22	29.5	1275.7	223−234[f]	1275.47
	29.5	1004.8	*h*	
23	30.2	3457.5	162−192	3457.85
	30.2	3074.8	165−192	3073.41
24	30.6	1414.7[d]	192−204	1414.71
25	30.8	1286.6	193−204	1286.54
	30.8	1060.6	*h*	
26	31.9	2084.3	74−93 or 205−222[e]	2084.43 or 2083.44
27	33.5	2675.7	**121−146 + 69.6 Da**[g]	2606.01
28[i]	35.8	2000.8	66−83	2000.52

[a] Peptides exhibiting a mass increment relative to unmodified TEM-2 peptides are highlighted in bold italics. Standard deviations were less than 1.2 Da from the mean reported mass. [b] Reported masses were obtained from the sum of all scans under the appropriate peak. [c] Assignments are quoted using the ABL numbering scheme (Ambler et al., 1991). [d] Masses were derived using a single charge state only. [e] The mass of peptide 74−83 is so similar to that of peptide 112−120 as to make the two indistinguishable. Peptides 74−93 and 205−222 are similarly indistinguishable. [f] The masses of peptides 56−65 and 223−234 are very similar but the presence of a strong $(M+3H)^{3+}$ peak under peak 10 alone suggests the above assignment since peptide 56−65 has an extra basic group. [g] Mass shift derived by subtracting calculated mass of unmodified peptide. [h] Mass not assigned. [i] A trace of peptide exhibiting a mass increment consistent with a 69.92 ± 0.04 mass increment to peptide 66−83 eluted in peak 28.

Glu-C in the presence of DTT, assignments for which are shown in Table 5. Modified peptides were observed in peaks 17, 19, and 23. The mass spectrum derived from peak 23 indicated the presence of four species. Those with masses of 3571.5 and 3589.5 Da correspond to peptide 59−79 modified by 69.5 and 87.5 Da, respectively. Similarly, those with masses of 5382.5 and 5403 correspond to peptide 59−104 modified by 70.5 and 91 Da, respectively. The similarity of these species to those obtained from experiments involving proteolytic digestion of fraction 2 of the partial purification procedure is apparent. Proteolytic digestion of fraction 1, however, consistently yielded a lesser amount of unmodified Ser-70 containing peptides and indeed is virtually absent (Figure 5). Peptides containing Cys-123 were present solely in the unmodified form. The mass spectrum derived from peak 18, Figure 5 is consistent with the presence of unmodified peptide 122−145. N-Terminal sequencing of the protein present in peak 18 afforded the sequence LCSAAITMXD, which is consistent with this assignment. Peaks 17 and 19, eluting either side of peak 18, both exhibited a mass spectra consistent with the assignment of dehydrated peptide 122−145, an assignment supported by N-terminal sequencing of peak 17 that yielded the same sequence as peak 18.

DISCUSSION

Intact ESIMS Studies. ESIMS studies on the modification of TEM-2 by clavulanate using intact protein led to the observation of new species corresponding to the production of four different mass modifications to TEM-2: B, C, D, and E corresponding to positive mass increments of 155, 52, 70, and 88 Da, respectively. From inspection of Table 1 it is apparent that only B is large enough to include the carbons of both β-lactam and oxazolidine rings of the clavulanate skeleton. Species B is nevertheless too small to represent structures such as the enamines **3a** and **3b**; the mass increment of 155 Da corresponds instead to a decarboxylated enamine or imine such as structure **6, 9**, or **13**,



consistent with prior proposals (Reading & Hepburn, 1978). It seems probable that species C, D, and E (Table 1) all contain the carbons of the β-lactam ring alone, and, of these, species C is consistent with either of the cross-linked structures **4** or **7**. We propose structures **10a** [see also Knowles (1980)] and **11a** as sensible candidates, in keeping with the observed masses of species D and E, that might arise from hydrolysis of imine **13** or enamine **9** or from hydrolysis of a cross-linked species such as enol ether **4** or imine **7** (Figure 6). Time course experiments were carried out following incubation of TEM-2 with *tert*-butylammonium clavulanate at an inhibitor/enzyme ratio of 18:1. These clearly demonstrated the return to predominance of unmodified TEM-2 and the broadly parallel declines in intensity of species D and E, consistent with the intermediacy of one or both of aldehyde **10a** and/or hydrated aldehyde **11a** in the major pathway for the regeneration of unmodified enzyme. Species B was observed to have a significantly shorter lifetime than D or E and is thus proposed as an intermediate in the inhibition and regeneration processes of TEM-2. In contrast the intensity of species C, corresponding to a putative cross-linked complex such as **4** or **7**, was observed to decline only slowly.

Low-resolution ESIMS experiments using intact protein are limited by their inability to distinguish between species

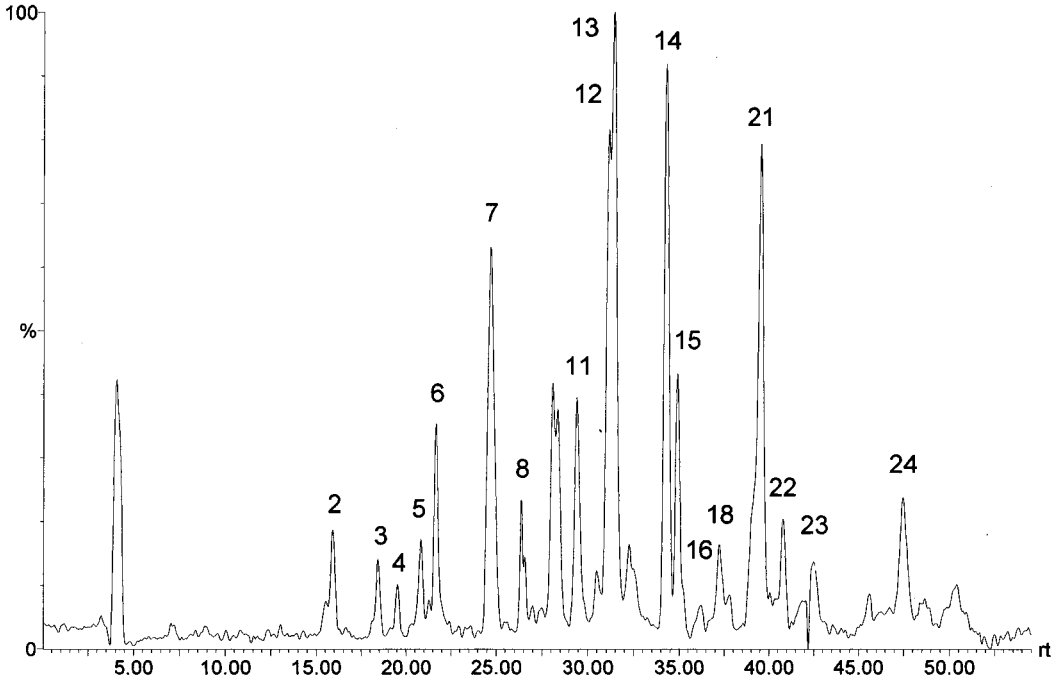


FIGURE 5: Total ion current chromatogram of partially purified clavulanate inhiibited TEM-2 β-lactamase (fraction 1) following digestion with endoproteinase Glu-C in the presence of DTT (6 h).

Table 5: Selected Assignments for HPLC-ESIMS Analysis of Partially Purified Clavulanate-Inhibited TEM-2 (Fraction 1) following Digestion with Endoproteinase Glu-C in the Presence of DTT (6 h)[a]

peak no.	retention time (min)	mass[b] (Da)	assignment[c]	calculated mass (Da)
1	15.5	1421.8	36−48	1420.59
14	34.3	2697.9	203−240	2698.08
16	36.1	6100.6	203−274	6097.95
17	36.8	***2589.7 ± 1.30***	**122−147 − 17 Da**[e]	2606.01
18	37.2	2606.3	122−147	2606.01
19	37.9	2588.3	**122−147 − 18 Da**[e]	2606.01
23	**42.5**[e]	3571.6	**59−79 + 69.5 Da**[d]	3502.10
		3589.7	**59−79 + 87.5 Da**[d]	3502.10
		5382.6	**59−104 + 70.5 Da**[d]	5312.10
		5402.9	**59−104 + 91 Da**[d]	5312.10

[a] Peptides exhibiting a mass change relative to unmodified TEM-2 peptides are highlighted in bold italics. Except where indicated in the third column, the standard deviations were less than 1 Da from the mean reported mass. [b] Reported masses were obtained from the sum of all scans below the appropriate peak. [c] Assignments are quoted using the ABL numbering scheme (Ambler et al., 1991). [d] Mass shift was derived by subtraction of the calculated mass of unmodified peptide. [e] Mass shift was derived by subtraction of the observed mass of unmodified peptide. In addition to the peaks assigned above peptides, peaks assigned as corresponding to peptides 275−281, 105−110, 59−64, 29−37, 111−121, 90−104, 49−58, 29−48, 282−290, 148−163, 241−273, 148−168, 198−212, 178−197, 170−197, 178−281, 172−274, and 172−281 and the Glu-C enodoproteinase were observed.

of identical, or indeed very similar, mass. Any of the above species might thus represent more than one modified protein. In addition, we cannot discount the possibility that further species, too weak to be clearly visible, were present, for example the unassigned peaks in Figure 2b. Care must also be taken in extrapolating results of *in vitro* studies to the physiological situation. Note, however, that the same mass increments were observed whether a low (18:1) or high (350:1) clavulanate/enzyme ratio was used.

Proteolysis and HPLC-ESIMS Studies. The location of the covalent modifications to the TEM-2 β-lactamase including the putative cross-linking residues was investigated using proteolysis followed by HPLC-ESIMS analysis. Interpretation of the results is complex, but they clearly demonstrate demonstrate that both Ser-70 and Ser-130 are covalently modified by clavulanate and thus it is the enol ether **7** which is the cross-linking species resulting from clavulanate inhibition of the TEM-2 β-lactamase.

Partial purification of clavulanate-inhibited TEM-2 by HPLC allowed separation of the inhibited material into two fractions (fraction 1 and fraction 2), which both contained mass increments of 52 and 70 Da relative to unmodified TEM-2. Subsequent proteolysis of these purified fractions followed by HPLC-ESIMS allowed the purification and on-line mass analysis of clavulanate-modified peptides from the vicinity of the active sites of both fractions 1 and 2. The predominant protein present in nascent fraction 1 was assigned as the aldehyde/hydrated aldehyde (**10b**/**11b**) linked to Ser-70 with a dehydroalanyl residue present at residue 130. The predominant protein present in nascent fraction 2 was assigned as the cross-linked vinyl ether **7**, which was shown to slowly degrade to fraction 1, i.e., aldehyde/hydrated aldehyde **10b**/**11b**. The evidence for these assignments is discussed below. It is also noteworthy that clavulanate-inhibited TEM-2 is considerably more resistant to proteolytic digestion than is the unmodified enzyme, presumably in part due to the cross linking in enol ether **7**.

The lower charge states involved in ESIMS of oligopeptides, as compared to intact proteins, affords an intrinsic precision advantage in the measurement of mass increments. More confidence can thus be placed in the accuracy of those mass shifts reported in Table 1 (intact protein ESIMS) that are subsequently confirmed in HPLC-ESIMS studies. Of further interest is the positional information afforded by this technique. The most striking conclusion is that, although the 70 Da mass increment present in the intact protein work on both fractions 1 and 2 was confirmed, there are clearly *two* types of modification resulting in this mass shift: it can arise between residues 66 and 79 (peak 19, Table 3 and peak

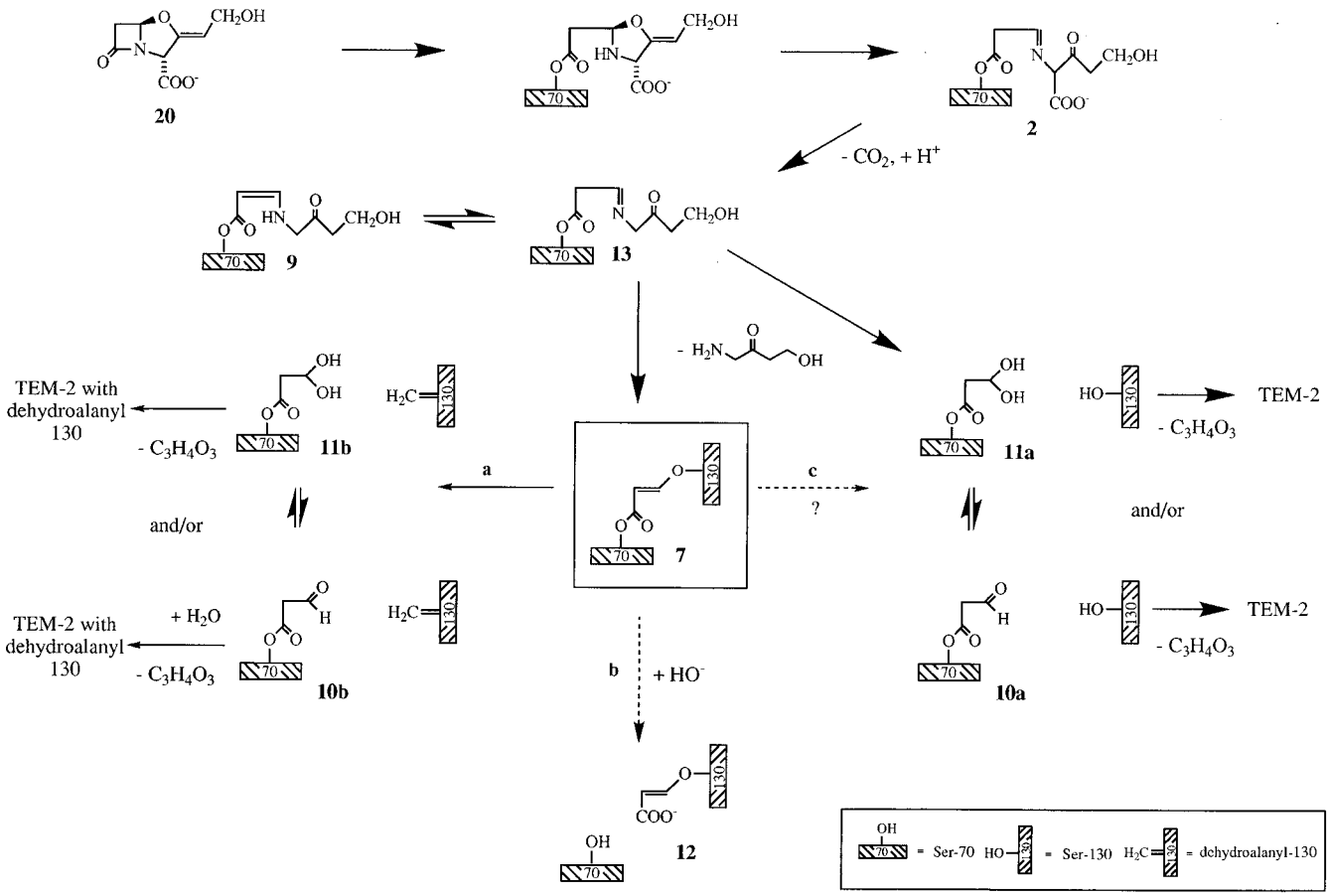


FIGURE 6: Mechanism for the reaction of TEM-2 β-lactamase with clavulante. Imine **13** is proposed as a key intermediate with may either form the stable cross linked vinyl ether **7** or be converted to the aldehyde/hydrated aldehyde **10a/11a**, one or both of which is hydrolyzed to give unmodified TEM-2. Also indicated are possible reaction pathways (a, b, c) for the vinyl ether **7.** Under the acidic conditions of the HPLC purification, **7** reacts via pathway a. Evidence for pathway b was accrued only after proteolytic digestion; thus ester hydrolysis may only occur after partial proteolysis. No direct evidence was accrued for pathway c, but partial hydrolysis via this pathway cannot be ruled out.

28, Table 4, digestion of fraction 2, and peak 23, digestion of fraction 1, Table 5) or between residues 122 and 146 (peaks 14 and 20, digestion of fraction 2, Table 3 and peak 27, digestion of fraction 2, Table 4). The former region, which was observed to be modified by mass increments of both 70 and 88 Da in experiments digesting both fractions 1 and 2 [Tables 5 (fraction 1) and 3 and 4 (fraction 2)] from the partial purification by HPLC, contains the known acylation site Ser-70. The second locus containing Ser-130 was observed to be modified only in those experiments involving digestion of fraction 2 and to be modified by 70 Da only, i.e., no mass increment of 88 Da was observed. Several potentially nucleophilic residues are located between residues 122 and 146, but of these only Ser-130 and Asp-131 are conserved and in close proximity to the active site (Moews et al., 1990). The side chain of Asp-131, however, is directed away from the catalytic residues (Moews et al., 1990) leaving Ser-130 as the most likely candidate for a nucleophilic role. Although covalent modification to peptides containng Ser-130 was only demonstrated after HPLC under acidic conditions, the detetction of these peptides exclusively in a single covalently modified form suggests that modification did indeed result from clavulanate inhibition rather than from the less specific reactions that might otherwise accompany exposure to the acidic conditions. The covalent involvement of Ser-130 in the inhibition mechanism strongly suggests that it is the enol ether **7** [see also Imtiaz et al. (1993)], rather than the enamine **4**, that represents the cross-linked species responsible for the 52 Da mass shift detected in intact protein work. Ester hydrolysis of enol ether **7** during proteolysis or purification would result in species **12**, consistent with the observed 70 Da (but not 88 Da) mass increment between residues 122 and 146 (after HPLC-ESMS of fraction 2). The simultaneous observation of unmodified Ser-70 containing peptides from the digestion of fraction 2 (note the relatively strong intensity of series B, Figure 4c) is in keeping with this proposal.

The observation of 70 and 88 Da mass increments in a peptides containing Ser-70 after proteolysis of both fractions 1 and 2 is consistent with the formation of the aldehyde/hydrated aldehydes (**10a**/**11a** and **10b**/**11b**). The presence of Ser-130 dehydrated forms of peptide 122−147 in the fraction 1 digestion experiments presumably reflects the observed production of apparently dehydrated TEM-2 from intact fraction 1. Since fraction 2 was observed to convert to fraction 1, it thus seems probable that the major pathway open to enol ether **7** prior to digestion is the elimination of water from the cross-linked Ser-130 to yield an (hydrated) aldehyde and a dehydroalanyl residue at residue 130 (**10b**, **11b,** Figure 6). Peptides containing apparently unmodified

Ser-130 were also observed by HPLC-ESIMS analysis after proteolysis of fraction 1 from the partial HPLC purification. These may have resulted from the presence of low levels of Ser-130 unmodified TEM-2, which were not visible in the intact protein studies on fraction 1, and/or by rehydration of the dehydroalanyl residue at position 130 to regenerate apparently unmodified Ser-130.

Experiments digesting fraction 2 from the partial purification procedure also yielded Ser-70 containing peptides exhibiting 70 and 88 Da mass increments, albeit in a smaller amount than observed from fraction 1. This observation is also consistent with the existence of a pathway from the cross-linked enol−ether **7** to the Ser-70 modified species **10b** and **11b** as shown in Figure 6. Note that the conversion of enol ether **7** to aldehyde/hydrated aldehyde **10a**/**11a** cannot be ruled out, but that there was no evidence for unmodified Ser-130 in the fraction 1 produced from fraction 2. The simultaneous apparent absence in these experiments of residue-130 containing peptides (especially dehydroalanyl), other than those consistent with structure **12** showing a 70 Da mass increment, may then seem curious. However, given their necessary low intensity, the possibility of multiple pathways from enol ether **7** to peptides containing various modifications at Ser-70 or Ser-130 under the proteolysis conditions, and the observed digestion related heterogeneity, their dilution to invisibility seems feasible. The cross-linked nature of enol ether **7** probably precluded its direct observation by HPLC-ESIMS after proteolysis by ensuring an unavoidable dilution. The observation of the undigested TEM-2 + 52 Da species (peak 22, Table 3) in analyses of fraction 2 digestions showing Ser-130 modification, and no evidence of unmodified or dehydrated Ser-130, is further evidence for the presence of species **7** in fraction 2 when species **10b**, formed by elimination from enol ether **7**, could explain an identical mass increment in the intact enzyme.

Mechanistic Conclusions. Figure 6 summarizes the proposals outlined above. Intact protein ESIMS demonstrated mass increments consistent either with the formation of either enamine **9** or imine **13,** aldehyde **10a,** hydrated aldehyde **11a**, and enol ether **7**. The failure to observe mass increments corresponding to proposed intermediates upto and including enamine **9** and imine **13** indicates that their formation is rapid. Attempted separation by HPLC of the clavulanate-inhibited TEM-2 species led to purification of two fractions (fraction 1 and fraction 2). The major species present in nascent fraction 2 was assigned as the cross-linked enol ether **7**. The major species present in nascent fraction 1 was assigned as TEM-2 modified with an (hydrated) aldehyde at Ser-70 and with a dehydroalanyl residue at 130 (**10b**/**11b**). The latter assignment indicates that the dehydration of Ser-130 to produce a dehydroalanyl residue has a significant effect on the rate of hydrolysis of the aldehyde/hydrated aldehyde at Ser-70, since time course studies indicate that aldehyde/hydrated aldehyde **10a**/**11a** are significantly more labile to hydrolysis than are aldehyde/hydrated aldehyde **10b**/**11b**. This is of interest with respect to proposed strutural and functional roles for Ser-130 in the hydrolysis of β-lactams by TEM-2 and other Class A β-lactamases [for a recent review, see Matagne and Frere (1995)]. The HPLC-ESMS studies provided direct evidence for modifications at Ser-70 and Ser-130 of inhibited TEM-2 and were supported by Edman sequencing of isolated peptides. The lability to hydrolysis of the aldehyde/hydrated aldehyde (**10a**/**11a**) and the even shorter lifetime of the species responsible for the 155 Da mass increment (imine **13** or enamine **9**) probably precluded their purification and characterization after proteolysis by HPLC-ESIMS. The HPLC-ESMS studies also indicated that the major pathways for the hydrolysis of enol ether **7** under the nonphysiological acidic conditions involves the unexpected production of a dehydroalanyl residue at residue-130 and a (hydrated) aldehyde at Ser-70 (**10b**, **11b,** Figure 6). It is possible that this unusual fragmentation of a vinyl ether reflects strain present in the cross-linked β-lactamase structure. Hydrolysis of the ester linkage present in aldehyde/hydrated aldehyde **10b**/**11b** is slow and results predominantly in the production of dehydrated TEM-2. It is unclear as to whether or not this unusual fragmentation pathway for a vinyl ether occurs under physiological conditions.

Time course studies using intact protein indicated that the major pathway for the regeneration of unmodifed TEM-2 after incubation with clavulanate is via aldehyde/hydrated aldehyde (**10a**/**11a)** (Ser-130 unmodified) and that cross-linked species **7** is relatively stable. It cannot be ruled out that under physiological conditions hydrolysis of enol ether **7** to aldehyde/hydrated aldehyde **10a**/**11a** via the usual vinyl ether hydrolysis mechanism occurs resulting in subsequent regeneration of unmodifed TEM-2. However, the available evidence, albeit accrued under nonphysiological conditions, implies the operation of such a process at a significant rate is unlikely. It instead appears that the major degradation pathway open to purified enol ether **7** proceeds via aldehyde/hydrated aldehyde **10b**/**11b**, which are stable enough to be purifed and characterized, and ultimately results, after ester hydrolysis, in the formation of TEM-2 dehydrated at residue 130. Thus, we propose that formation of the cross-linked species **7** results in the effective complete inactivation of TEM-2. It follows that minimization of the conversion of imine **9** and enamine **13** to aldehyde/hydrated aldehyde **10a**/**11a** and maximization of conversion to enol ether **7** is one potential strategy for enhancing the potency of clavulanate.

ACKNOWLEDGMENT

We thank Drs. C. V. Robinson, P. L. Roach, and C. H. Frydrych and colleagues for helpful discussions and encouragement, A. C. Willis for N-terminal sequencing, C. R. A. Kirkup (Public Health Laboratory Service Centre for Applied Microbiology and Research) for providing *E. coli* TEM-2 β-lactamase, and the E.P.S.R.C., M.R.C., B.B.S.R.C., and SmithKline Beecham Pharmaceuticals for financial support of this work.

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BI961044G