Identification of Histidine Residues that Act as Zinc Ligands in β -Lactamase II by Differential Tritium Exchange

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1. Four histidine-containing peptides have been isolated from a tryptic digest of the Zn^{2+} -requiring β -lactamase II from *Bacillus cereus*. One of these peptides probably contains two histidine residues. 2. The presence of one equivalent of Zn^{2+} substantially decreases the rate of exchange of the C-2 proton in at least two and probably three of the histidine residues of these peptides for solvent ^3H . 3. It is concluded that peptides containing at least two of the three histidine residues acting as Zn^{2+} ligands at the tighter Zn^{2+} -binding site of β -lactamase II have been identified.

Bacillus cereus 569/H/9 produces two extracellular β -lactamases that differ in their rates of hydrolysis of the β -lactam ring of penicillins and cephalosporins, and in their physical properties (Davies *et al.*, 1974). Perhaps the most striking difference between the two enzymes is the requirement of a metal ion for β -lactamase II activity (Sabath & Abraham, 1966); activity is highest with the Zn^{2+} -requiring enzyme (Davies & Abraham, 1974).

β -Lactamase II has been shown to bind two Zn^{2+} ions by equilibrium dialysis (Davies & Abraham, 1974) and n.m.r. spectroscopy (Baldwin *et al.*, 1978). Hydrolysis of all substrates studied parallels the binding of the first Zn^{2+} ion (Davies *et al.*, 1974; M. Brightwell, unpublished results). The ligands at the first metal-binding site may consist of the thiol group of the enzyme's sole cysteine residue (Davies & Abraham, 1974) and three of the enzyme's five histidine residues (Baldwin *et al.*, 1978). The observation that the presence of one equivalent of Zn^{2+} substantially decreased the exchange rate of the C-2 protons of these three histidine residues for solvent ^2H (Baldwin *et al.*, 1978) suggested that ^3H exchange (Matsuo *et al.*, 1972; Ohe *et al.*, 1974) could be used to identify directly the three histidine residues that act as Zn^{2+} ligands at the first Zn^{2+} -binding site. The results of such an experiment are reported in the present paper.

Materials and Methods

Materials:

β -Lactamase II was isolated by the method of Davies *et al.* (1974). $^3\text{H}_2\text{O}$ (5 Ci/ml) and iodo[2- ^{14}C]-acetic acid (sp. radioactivity 57 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. The iodo[2- ^{14}C]acetic acid was dissolved in 250 μl of 200 mM-iodoacetic acid (recrystallized from light

petroleum, b.p. 60–80°C) to give a solution of sp. radioactivity 0.98 mCi/mmol. Guanidinium chloride (AristaR grade) was from BDH, Poole, Dorset, U.K., trypsin was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. and Dextran Blue 2000 was from Pharmacia, Uppsala, Sweden. Other chemicals were AnalaR grade.

^3H exchange

Samples of β -lactamase II were dialysed against 1 litre of 20 mM-sodium succinate, 20 mM-triethanolamine/HCl, 1 M-NaCl, pH 8.05, containing 10 mM-EDTA (350 μl , 4.5 mM-enzyme) or 50 μM - ZnSO_4 (250 μl , 4.5 mM-enzyme) and then placed in Pyrex tubes (7.5 cm $\times$ 0.8 cm). Calculations based on dissociation constants of 2.36 μM (pH 6.0, 30°C, 1 M-NaCl) (M. Brightwell, unpublished work) and 24 mM (pH 5.7, 26.5°C, 1 M-NaCl) (Baldwin *et al.*, 1978) for the first and second Zn^{2+} sites respectively indicate that this concentration of Zn^{2+} will result in 96% occupancy of the first site and 0.2% occupancy of the second site at pH 6. After the addition of 0.1 vol. of $^3\text{H}_2\text{O}$ (5 Ci/ml) the tubes were sealed and incubated at 37°C for 208 h. In a separate experiment in which water replaced $^3\text{H}_2\text{O}$ it was established that 11% of the activity of a solution of the mono-zinc enzyme and 28% of that of the apoenzyme were lost in precipitates formed under these conditions. Precipitated protein was removed by centrifugation, and washed with 2.5 ml of water. The initial supernatants were combined with the corresponding washings, dialysed twice at 4°C against 5 litres of 15 μM - ZnSO_4 , pH 4.9, and the solution then freeze-dried.

Carboxymethylation

Samples (25 mg) of Zn^{2+} - β -lactamase II and apo-(β -lactamase II) following ^3H exchange were each

dissolved in 1 ml of a solution containing 100 mM-Tris/HCl, 1 mM-dithiothreitol and 5 M-guanidinium chloride, pH 8.58. After N_2 had been bubbled through the solutions for 1 h, 50 μ l of 200 mM-iodo[2- 14 C]acetic acid (sp. radioactivity 0.98 μ Ci/ μ mol; 3-fold excess over enzyme plus dithiothreitol) was added to each solution. The reaction was allowed to proceed for 1 h in the dark under N_2 and was then stopped by addition of 20 μ l of 2-mercaptoethanol (20-fold excess over iodoacetic acid). The solutions were dialysed twice at 4°C against 8 litres of water, pH 4.9, and once against 4 litres of 0.5% (w/v) NH_4HCO_3 .

Tryptic digestion

Each dialysed solution was made up to a total volume of 5 ml with 0.5% NH_4HCO_3 . Trypsin (50 μ l of a solution containing 5 mg of enzyme/ml of 1 mM-HCl) was added, and digestion allowed to proceed for 2 h at 37°C. A further 50 μ l of the trypsin solution was added, and after 2 h at 37°C the solutions were freeze-dried.

Gel filtration on Sephadex G-25

The tryptic digests (25 mg) were each dissolved in 0.5 ml of 0.5% (w/v) NH_4HCO_3 containing 10% (v/v) propan-2-ol, and applied to a column (137 cm $\times$ 1 cm) of Sephadex G-25, previously equilibrated with the same solution. Elution was carried out at a flow rate of 5.7 ml/h and 1.9 ml fractions were collected.

Paper electrophoresis and chromatography

Peptides were further purified by electrophoresis (45 min, 60 V/cm) at pH 6.5 and 4.5 in the buffers described by Ambler (1963), and by chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.; solvent A). Radioactive peptides were detected by fluorography (Laskey & Mills, 1975). Unlabelled peptides were detected with Cd^{2+} -ninhydrin (Heilmann *et al.*, 1957), or with the Pauly reagent (Offord, 1969). The purity of peptides after elution with 1 mM- NH_3 was checked by electrophoresis (45 min, 60 V/cm) at pH 1.8.

Amino acid analysis

Peptides were hydrolysed with 6 M-HCl containing 1% (w/v) phenol in evacuated tubes for 20 h at 110°C unless otherwise stated. Amino acid analyses were carried out by a single column procedure on an LKB Biochrom 4101 analyser (LKB Instruments, South Croydon, Surrey, U.K.). No correction was made for destruction of threonine and serine. Determinations of *N*-terminal amino acid residues were made by the dansyl method (Gray, 1972).

Determination of radioactivity

The radioactivities of samples were determined in a total volume of 250 μ l of water and 3 ml of Unisolve 1 scintillation fluid (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) with a Nuclear-Chicago Unilux IIA scintillation counter (G. D. Searle, Nuclear-Chicago Division, High Wycombe, Bucks., U.K.).

Results and Discussion

Incorporation of radioactivity

The extent of incorporation of 3H at the C-2 position of the histidine residues of Zn^{2+} - β -lactamase II and apo-(β -lactamase II) during 3H exchange is shown in Table 1. The values of 1.8 and 3.3 mol of 3H /mol of β -lactamase II for the Zn^{2+} -enzyme and the apoenzyme respectively are both lower than the values of 2.5 and 5 mol of 2H /mol of enzyme obtained for 2H exchange under the same conditions in the n.m.r. study described by Baldwin *et al.* (1978). This discrepancy may arise from partial back exchange during the separation of the enzyme from excess 3H_2O by dialysis, or during carboxymethylation, or both.

The extent of incorporation of ^{14}C during carboxymethylation is lower than the value of 0.72 mol of ^{14}C /mol of β -lactamase II, reported by Davies *et al.* (1974). However in a separate experiment under the

Table 1. Incorporation of radioactivity into β -lactamase II by 3H exchange

After 3H exchange in the presence and absence of Zn^{2+} , samples of β -lactamase II were carboxymethylated by reaction with iodo[2- ^{14}C]acetate, as described in the Materials and Methods section, before tryptic digestion. Protein was determined by measurement of u.v. absorption, a value of 9.3 being used for $A_{1\text{cm}}^{1\%}$ at 280 nm (Davies, 1973). The molecular weight of the enzyme was taken to be 22000 (Davies *et al.*, 1974).

	^{14}C or 3H radioactivity in S-[^{14}C]carboxymethyl- β -lactamase II (mol/mol of enzyme)			
	Before tryptic digestion		After tryptic digestion	
	3H	^{14}C	3H	^{14}C
Zn^{2+} -enzyme	1.86	0.51	1.75	0.54
Apoenzyme	3.30	0.30	3.32	0.37

same conditions a value of 0.67 mol of ^{14}C /mol of enzyme was obtained, and carboxymethylcysteine was the only radioactive compound observed after electrophoresis at pH 1.8 of an acid hydrolysate of the product. It is noteworthy that the value of 1 mol of thiol group reacted/mol of β -lactamase II reported for the reaction between Ellman's reagent and β -lactamase II in 5M-guanidinium chloride (Davies & Abraham, 1974) was based on a molar absorption coefficient of 11000 for the 2-nitro-5-thiobenzoate anion (Davies, 1973). Substitution of the correct value of 13600 (Ellman, 1959; Gething & Davidson 1972) yields a value of 0.81 mol of thiol group/mol of enzyme. Subsequent experiments have routinely yielded values between 0.7 and 0.8 mol of thiol group/mol of enzyme.

Fractionation of tryptic peptides from β -lactamase II

Partial separation of groups of tryptic peptides was achieved by gel filtration on a column of Sephadex G-25, and the peptides revealed by the ^3H and ^{14}C -

content and the absorption at 280nm of the filtrate. Staining with the Pauly reagent after electrophoresis at pH 6.5 of samples (50 μl) from every second fraction of the filtrate revealed four major groups of orange-red spots in fractions 28–39, one at the origin (peptide 4) and three at increasing distances towards the cathode (peptides 3, 2 and 1 respectively), which were later shown to be histidine-containing peptides, and one major group of purple spots in fractions 42–46, which was later shown to be due to a peptide containing tyrosine residues, but no histidine residues. Fractions 28–39 were pooled, and the histidine-containing peptides were further purified by electrophoresis at pH 6.5 and chromatography in solvent A; their amino acid compositions are given in Table 2 and their properties are summarized in Table 3. Peptides 1 and 2 appeared to be pure after these steps, as judged by the absence of other ninhydrin-positive material after electrophoresis at pH 1.8, and by the presence of a single *N*-terminal amino acid. It was found convenient to locate peptide 2 with the Pauly reagent rather than Cd^{2+} -ninhydrin. Peptide 3 some-

Table 2. *Amino acid composition of histidine peptides from β -lactamase II*

Peptides, numbered as in the text, were isolated from a tryptic digest of β -lactamase II tritiated in the presence (Z) or absence (A) of Zn^{2+} , or from untritiated β -lactamase II (U). Peptides were hydrolysed for 20h under the conditions described in the Materials and Methods section, with the exception of peptide 2 (Z), which was hydrolysed for 48h. Peptide compositions are given as mol of amino acid/mol of peptide. The value for mol of peptide was obtained by adding the amounts of all amino acids other than suspected contaminants (i.e. those amino acids present at less than 0.4 mol/mol of peptide, which values are shown in parentheses), and valine and isoleucine. Valine and isoleucine were omitted because they are often released slowly. The sum was then divided by the most probable number of residues other than valine and isoleucine, which value is given in parentheses at the foot of the Table. Peptide 4 stained purple with Ehrlich's reagent (Offord, 1969) and is therefore presumed to contain tryptophan.

Amino acid	Peptide composition (mol of amino acid/mol of peptide)											
	1			2			3			4		
	Z	A	U	Z	A	U	Z	A	U	Z	A	U
Asp				2.2	2.1	2.0	2.6	2.9	3.0	4.2	4.8	6.5
Thr	2.0	1.9	2.0	1.9	2.1	1.7	(0.1)			2.7	2.3	2.4
Ser	1.0	1.0	1.1	(0.2)	(0.4)		(0.1)			2.8	3.0	4.0
Glu	1.2	1.2	1.2	(0.2)	(0.2)		1.3	1.1	1.1	2.8	2.3	1.8
Pro							1.2	1.1	1.1	1.4	1.2	0.7
Gly				(0.3)	(0.2)		2.7	2.8	3.0	3.4	3.2	3.0
Ala	3.7	3.7	3.8	2.0	1.9	1.8	1.0	1.0	1.0	0.9	0.9	0.8
Val				1.2	1.4	1.3	2.4	2.4	2.1	5.0	4.8	3.8
Met										(0.1)	(0.3)	
Ile				1.7	1.2	1.0	1.1	1.0	0.8	0.8	0.6	
Leu	2.0	2.1	2.1							4.2	4.3	4.9
Tyr										0.6	0.4	
Phe										0.9	0.8	0.7
His	1.0	1.1	1.0	1.8	2.0	1.3	1.1	1.0	1.0	1.0	1.1	1.0
Lys	1.1	1.1	1.0				1.1	1.1	1.0	2.4	2.1	2.0
Arg				1.0	0.9	1.0				(0.1)		
Trp												
nmol of peptide	26.4	28.1	25.4	9.1	3.2	17.6	30.0	17.7	28.7	32.0	23.8	8.1
Number of residues	12	12	12	12	11	11	14	14	14	33	32	32
	(12)	(12)	(12)	(9)	(9)	(9)	(11)	(11)	(11)	(27)	(26)	(28)

Table 3. *Properties of histidine peptides from β -lactamase II*

Peptides, numbered as in the text, were isolated from β -lactamase II tritiated in the presence (Z) or absence (A) of Zn^{2+} , or from untritiated β -lactamase II (U). Determinations of *N*-terminal amino acids were made by the dansyl method (Gray, 1972). The initial yellow-orange colour obtained on reaction of peptide 3 with Cd^{2+} -ninhydrin is taken to indicate the occurrence of the *N*-terminal Asx as the amide Asn. The colours obtained on reaction with Cd^{2+} -ninhydrin are indicated by: R, red; SR, slowly developing red; YO $\rightarrow$ R, yellow-orange changing to red. Electrophoretic mobilities (*m*) are relative to aspartic acid (= -1.00) at pH 6.5, and serine (= 1.00) at pH 1.8.

Peptide ...	Properties of peptides											
	1			2			3			4		
	Z	A	U	Z	A	U	Z	A	U	Z	A	U
<i>N</i> terminal		Ala			Val			Asn			Gly	
Ninhydrin		R			SR			YO $\rightarrow$ R			YO $\rightarrow$ R	
Yield (%)	27	21	23	2.6	2.5	4.1	15	6.5	15	7.7	6.0	0.6
<i>m</i> _{6.5}	0.19	0.19	0.19	0.11	0.11	0.11	-0.06	-0.06	-0.09		Origin	
<i>m</i> _{1.8}	0.98	1.00	N.D.	1.06	1.06	N.D.	0.89	0.90	N.D.		Streaks	
<i>R</i> _{Xylene Cyanol} in solvent A	0.68	0.68	0.69	0.57	0.57	0.58	0.41	0.41	0.43	0.82	0.83	0.73

times required a further electrophoresis step at pH 1.8 to achieve purity.

The material referred to as peptide 4 presented some problems. In a trial run with unlabelled enzyme it was subjected to further steps of electrophoresis at pH 1.8 and 4.5. Considerable 'streaking' occurred at both these pH values, so the final assessment of purity rests on the single *N*-terminus detected. The uncertainty over the purity of this peptide is not considered to be a serious problem, since the specific radioactivities observed for the peptide isolated from Zn^{2+} -enzyme and apoenzyme did not indicate that the histidine residue it contains was one of the ligands at the first Zn^{2+} -binding site. The peptide was also difficult to remove from the paper; elution was finally achieved with solvent A. Because of the decrease in yield that these steps entailed, the peptide from tryptic digests of ^3H -exchanged β -lactamase II was eluted with solvent A after the chromatographic step. The paper was first eluted with 1 mM- NH_3 to remove any contamination peptides.

Assignment of histidine ligands

The probable compositions of the histidine-containing peptides from tryptic digests of carboxy-

methyl- β -lactamase II are given in Fig. 1. The compositions are such that none of these four peptides could have arisen by further tryptic digestion of any of the other peptides, provided that tryptic cleavage occurred only at lysine and arginine residues. For example, peptide 2 contains an arginine residue, which is absent from the other three peptides, and peptide 1 contains four alanine residues, whereas peptides 3 and 4 contain only one. Although all the amino acids present in peptide 3, with the exception of the isoleucine residue, are also present in peptide 4, the specific radioactivities of the two peptides isolated from the digest of the apoenzyme are quite different. Thus the histidine residues observed in these four peptides are all distinct. The probable total of five histidine residues observed is not inconsistent with the amino acid analysis, which suggests five (Davies *et al.*, 1974) or six (Thatcher, 1975) histidine residues. Five histidine resonances were observed in the n.m.r. spectrum of the enzyme (Baldwin *et al.*, 1978). However, a peptide containing a sixth histidine residue could have been present in the radioactive material that remained at the origin after electrophoresis at pH 6.5 and chromatography.

The specific radioactivities observed after ^3H exchange at the C-2 position of the histidine residues

Peptide	Amino acid composition
Non-ligand histidine residues	
1	Ala (Thr ₂ , Ser, Glx, Ala ₃ , Leu ₂ , His) Lys
4	Gly (Asx ₄₋₅ , Thr ₂₋₃ , Ser ₃ , Glx ₂ , Gly ₃ , Ala, Val ₄₋₅ , Leu ₄₋₅ , Phe, His, Lys, Trp) Lys
Ligand histidine residues	
2	Val (Asx ₂ , Thr ₂ , Ala ₂ , Val, Ile ₁₋₂ , His ₁₋₂) Arg
3	Asn (Asx ₂ , Glx, Pro, Gly ₃ , Ala, Val ₂ , Ile, His) Lys

Fig. 1. *Histidine peptides from β -lactamase II*

Determinations of *N*-terminal amino acid residues were made by the dansyl method (Gray, 1972). The placement of lysine and arginine as C-terminal residues is based solely on the specificity of trypsin.

Table 4. *Specific radioactivities of histidine peptides from tritiated β -lactamase II*
Peptides are numbered as in Fig. 1.

Peptide	Sp. radioactivity (^3H d.p.m./nmol of peptide)	
	Zn $^{2+}$ -enzyme	Apoenzyme
1	4970	4855
2 (1-2 histidine residues)	251	3980
3	508	5993
4	702	1745
Solvent	6867	7682

of β -lactamase II in the presence and absence of Zn $^{2+}$ are given in Table 4. It is apparent that the presence of one equivalent of Zn $^{2+}$ substantially decreases the exchange rate at three histidine residues, two in peptide 2 and one in peptide 3. A similar conclusion was reached previously from an n.m.r. study (Baldwin *et al.*, 1978) of the ^2H exchange of the C-2 histidine protons of β -lactamase II, where the presence of one equivalent of Zn $^{2+}$ decreased the rate of disappearance of three resonances referred to as B, C and D. Thus these histidine resonances in the n.m.r. spectrum of Zn $^{2+}$ - β -lactamase II probably correspond to the histidine residues in peptides 2 and 3, and these are ligands at the first Zn $^{2+}$ -binding site.

The specific radioactivity of peptide 4 is also dependent on the presence of one equivalent of Zn $^{2+}$, but the difference between Zn $^{2+}$ -enzyme and apo-enzyme samples is significantly less than with peptides 2 and 3. A similar situation was observed in the n.m.r. study, where one resonance, referred to as E, disappeared completely from the spectrum of apo-enzyme when this enzyme was kept in $^2\text{H}_2\text{O}$ for 9 days at pH 8.05, but was still apparent, though with greatly decreased intensity, in the spectrum of Zn $^{2+}$ -enzyme that had been kept for 11 days under the same conditions (Baldwin *et al.*, 1978). The position of resonance E was affected by the binding of a second equivalent of Zn $^{2+}$ to the enzyme, and it was concluded that histidine E was acting as a ligand at the second Zn $^{2+}$ -binding site. Appreciable occupancy of the second site, as suggested by incomplete ^2H and ^3H exchange in the presence of Zn $^{2+}$, may be expected at pH 8.0. Resonance E may now be assigned to the histidine residue in peptide 4.

This assignment is also consistent with the assignment of a further resonance, referred to as A, in the n.m.r. spectrum of β -lactamase II to the histidine residue in peptide 1. Resonance A was observed to disappear completely from the spectra of both apo-enzyme and Zn $^{2+}$ -enzyme during ^2H exchange over

a period of 11 days at pH 8.05. This agrees with the close similarities of the specific radioactivities of peptide 1 after ^3H exchange in the presence and absence of one equivalent of Zn $^{2+}$.

The work described in the present paper will enable the histidine residues that act as Zn $^{2+}$ ligands at the first Zn $^{2+}$ -binding site of β -lactamase II to be recognized when the complete sequence is known.

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References

- Ambler, R. P. (1963) *Biochem. J.* **89**, 349-378
- Baldwin, G. S., Galdes, A., Hill, H. A. O., Smith, B. E., Waley, S. G. & Abraham, E. P. (1978) *Biochem. J.* **175**, 441-447
- Davies, R. B. (1973) D. Phil. Thesis, University of Oxford
- Davies R. B. & Abraham, E. P. (1974) *Biochem. J.* **143**, 129-135
- Davies, R. B., Abraham, E. P. & Melling, J. (1974) *Biochem. J.* **143**, 115-127
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70-77
- Gething, M. J. H. & Davidson, B. E. (1972) *Eur. J. Biochem.* **30**, 352-353
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121-138
- Heilmann, J., Barollier, J. & Watzke, E. (1957) *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219-220
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335-341
- Matsuo, H., Ohe, M., Sakiyama, F. & Narita, K. (1972) *J. Biochem. (Tokyo)* **72**, 1057-1060
- Offord, R. E. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M., eds.), 2nd edn., p. 530, Clarendon Press, Oxford
- Ohe, M., Matsuo, H., Sakiyama, F. & Narita, K. (1974) *J. Biochem. (Tokyo)* **75**, 1197-1200
- Sabath, L. D. & Abraham, E. P. (1966) *Biochem. J.* **98**, 11c-13c
- Thatcher, D. R. (1975) *Methods Enzymol.* **43**, 640-652