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Histidine Residues as Zinc Ligands in β-Lactamase II

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1. The Zn(II)-requiring β-lactamase from *Bacillus cereus* 569/H/9, which has two zinc-binding sites, was examined by 270MHz ^1H n.m.r. spectroscopy. Resonances were assigned to five histidine residues. 2. Resonances attributed to three of the histidine residues in the apoenzyme shift on the addition of one equivalent of Zn(II). 3. Although these three histidine residues are free to titrate in the apoenzyme, none of them titrates over the pH* range 6.0–9.0 in the mono-zinc enzyme. 4. The ability of the C-2 protons of these three histidine residues to exchange with solvent (^2H_2O) is markedly decreased on Zn(II) binding. 5. It is proposed that these three histidine residues act as zinc ligands at the tighter zinc-binding site. 6. Resonances attributed to a fourth histidine residue shift on addition of further zinc to the mono-zinc enzyme. It is proposed that this histidine residue acts as a Zn(II) ligand at the second zinc-binding site.

Bacillus cereus 569/H/9 produces two extracellular β-lactamases. Enzymes of this family (EC 3.5.2.6) hydrolyse the β-lactam ring of penicillins or cephalosporins. β-Lactamase I is similar in molecular weight, substrate profile and amino acid sequence to the β-lactamases from two other Gram-positive organisms, *Staphylococcus aureus* and *Bacillus licheniformis* (Ambler & Meadway, 1969; Thatcher, 1975*a*). β-Lactamase II has a lower molecular weight (22000), a greater thermostability and a broader substrate profile (Crompton *et al.*, 1962; Davies *et al.*, 1974, 1975) and also differs from β-lactamase I in containing one cysteine residue (Kuwabara *et al.*, 1970; Davies *et al.*, 1974).

β-Lactamase II appears to be unique among the known β-lactamases in its requirement for a metal ion for activity (Sabath & Abraham, 1966). The highest activity has been obtained with zinc(II) enzyme, although the cobalt(II) enzyme is also active (Davies & Abraham, 1974). Equilibrium dialysis and kinetic studies have indicated that the enzyme can bind two zinc(II) ions and that hydrolysis of benzylpenicillin is dependent on metal binding at the site of higher affinity, whereas hydrolysis of cephalosporin C may also be affected by binding at the weaker site (Davies & Abraham, 1974). The spectra of the Co(II) and Cd(II) enzymes implicate the sole cysteine residue in metal binding (Davies & Abraham, 1974), but nothing has hitherto been published about the other metal ligands. The present n.m.r. study was undertaken to investigate metal-ion binding in greater detail.

Materials and Methods

Materials

β-Lactamase II was isolated by the method of Davies *et al.* (1974), and stored as a freeze-dried solid after dialysis against 5mM-NH_4HCO_3 containing 15μM-$ZnSO_4$. ^2HCl (99.0% ^2H), NaO^2H (99.0% ^2H) and ^2H_2O (99.8% ^2H) were from Ryvan Chemical Co., Southampton, U.K. All other chemicals were of AnalaR grade and were freeze-dried from ^2H_2O before use.

Preparation of samples

Apoenzyme. Freeze-dried β-lactamase II was dissolved in ice-cold ^2H_2O (1ml or less containing 1M-NaCl) to give a final enzyme concentration of 50–120mg/ml. EDTA in ^2H_2O (0.1vol. of 200mM, pH*6.0) was added and the sample left at 4°C for at least 16h to allow formation of the apoenzyme (pH* refers to the meter reading in ^2H_2O, the meter having been calibrated with buffers in water at 22°C). The concentration of EDTA was decreased to 10mM by dialysis at 4°C against 2×10ml of a buffer containing 20mM-sodium succinate, 20mM-triethanolamine/^2HCl, 10mM-EDTA, 1M-NaCl in ^2H_2O adjusted to the appropriate pH* with NaO^2H.

When samples of apoenzyme were to be used for titrations of the first zinc site the concentration of EDTA was lowered by successive dialyses against 10ml of 1M-NaCl, and 2×10ml of succinate/

triethanolamine/NaCl buffer containing 0.1mM-EDTA in ^2H_2O. The concentration of EDTA used to prevent binding of traces of adventitious metal ions was only 2% of the concentration of the enzyme.

Mono-zinc enzyme. Freeze-dried β-lactamase II, containing at least one equivalent of Zn(II), was dissolved in ice-cold ^2H_2O (1ml or less containing 1M-NaCl) to give a final enzyme concentration of 50–120mg/ml. The sample was then dialysed against 2×10ml of succinate/triethanolamine/NaCl buffer (usually at pH* 6.0) containing 50μM-$ZnSO_4$. Calculation based on dissociation constants of 2.36μM (pH6.0, 30°C, 1M-NaCl) (M. Brightwell, unpublished work) and 24mM (pH*5.7, 26.5°C, 1M-NaCl) (the present paper) for the first and second Zn(II) sites respectively indicates that this treatment will result in 96% occupancy of the first site, and 0.2% occupancy of the second site.

The enzyme was assayed at pH6.0 as previously described with benzylpenicillin as substrate (Davies *et al.*, 1974) before and after the n.m.r. experiments.

Deuterium exchange with β-lactamase II. β-Lactamase II (11mg/ml in buffer containing 20mM-Tris/^{2}HCl, 1mM-$ZnSO_4$, 1M-NaCl, in ^2H_2O, pH* 7.0) was treated with 5M-guanidinium chloride and 5mM-dithiothreitol for 30min at 4°C. The enzyme was refolded by dialysis against 5×100ml of the same buffer, 5×100ml of 1mM-$ZnSO_4$ in ^2H_2O, and finally 2×100ml of 5mM-NH_4HCO_3 in ^2H_2O. The specific activity of the final product was virtually identical with that of the enzyme before unfolding and refolding. The deuterium-exchanged enzyme was stored as a freeze-dried solid. The apo- and mono-zinc forms of the deuterium-exchanged enzyme were prepared as described under 'Preparation of samples'.

^1H n.m.r. experiments

All spectra were obtained at a frequency of 270MHz with a modified Bruker console, 6.4T superconducting magnet (Oxford Instruments) and Nicolet 1085 computer. Routinely, 1024 free induction decays were accumulated in 4096 data points, over a spectral width of 4kHz, with a 70° pulse and a 0.6s separation between consecutive pulses. A longer pulse-to-pulse separation (5.0s) was used in the accumulation of spectra of the refolded deuterium-exchanged enzyme samples so as to achieve reliable relative-intensity information. The residual water signal was suppressed by applying a pulse at the appropriate frequency at all times except during data acquisition (Campbell *et al.*, 1977). Difference spectra and convolution difference spectra (Campbell *et al.*, 1974) were used to aid resolution. These techniques make possible the observation of peaks otherwise obscured by underlying resonances. Chemical shifts are reported relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate as an internal standard.

Typically samples for n.m.r. experiments contained 4–5mM-enzyme, 20mM-succinate, 20mM-triethanolamine and 1M-NaCl in 0.4ml of ^2H_2O. Under these conditions the mono-zinc enzyme was stable to 70°C, but the apoenzyme was much less heat-stable. Spectra were therefore collected at 26.5°C to facilitate comparison between the apo- and the mono-zinc enzymes.

In pH-titration experiments it was better to alter the pH with a solution of triethanolamine in ^2H_2O (1:5, v/v), since adjustments with a saturated solution of succinic acid in ^2H_2O or with NaO^2H or ^2HCl caused some protein precipitation. The pH* was measured with a Pye-Ingold micro-electrode (Instrumentation Laboratories, Altrincham, Cheshire, U.K.) fitted to a Radiometer pH-meter 26 (Radiometer, Copenhagen, Denmark), calibrated with potassium hydrogen phthalate and disodium tetraborate standard buffers. Maximum chemical-shift differences between protonated and unprotonated imidazole rings, and the pK_a* values for the histidine residues, were obtained from direct linear plots as described by Browne *et al.* (1976).

In zinc-titration experiments the pH* was measured after each zinc addition and adjusted upwards as necessary. It is estimated from measurements of enzyme activity that about 1% of the solution was lost, as electrode washings, per pH* measurement. The concentrations of enzyme and zinc were calculated on this basis.

Results

The 270MHz ^{1}H n.m.r. spectrum of β-lactamase II in ^2H_2O in the presence of one equivalent of Zn(II) (the mono-zinc enzyme) is shown in Fig. 1. It is typical of that expected for a protein of mol.wt. 22000, except that the number of resonances from amide protons in the region 7–11p.p.m. is small. The resonances to high field of the suppressed solvent HO^2H resonance (at 4.8p.p.m.) are due to the numerous methine, methylene and methyl groups. At highest field (near 0p.p.m.) a small number of ring-current-shifted methyl-group resonances are apparent. To low field of the HO^2H resonance the major intensity (6.5–8p.p.m.) is due to the ring protons of phenylalanine, tyrosine and tryptophan residues as well as histidine C-4 protons (I). Histidine C-2 protons appear in the region of 7.5–9p.p.m. The considerable resolution enhancement achieved by the convolution difference technique is shown in Fig. 1(*c*).

Recognition of histidine resonances

Of the two ring protons of histidine residues the C-2 proton gives a resonance between 7.5 and

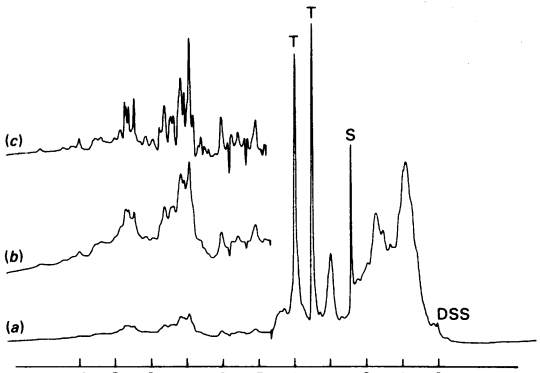
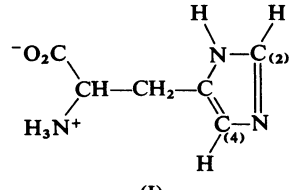


Fig. 1. *270MHz ^1H n.m.r. spectrum of mono-zinc β-lactamase II*

The enzyme (5.5mM) was in 20mM-succinate/20mM-triethanolamine/1M-NaCl, pH* 6.86 at 26.5°C. Resonances marked DSS, S and T are from the internal standard sodium 2,2-dimethyl-2-silapentane-5-sulphonate, succinate and triethanolamine respectively. (*a*) Conventional spectrum; (*b*) aromatic region of (*a*) with a vertical scale expansion of 4; (*c*) the convolution difference spectrum of the aromatic region.



(I)

9p.p.m., and the C-4 proton between 6.6 and 7.5p.p.m. In the absence of exchange broadening these resonances are often sharp. The resonances of both protons may be recognized in histidine residues, which are free to titrate by their downfield shift as the imidazole ring becomes protonated (Markley, 1975). In addition the C-2 proton may often be recognized by its ability to exchange slowly with solvent deuterium under certain conditions. In some cases identification by these procedures may be complicated by the presence of resonances from amide protons of the polypeptide backbone in the same region.

Spectra of deuterium-exchanged enzyme

The aromatic region of the spectrum of mono-zinc β-lactamase II is considerably simplified when the enzyme used has been unfolded and then refolded in

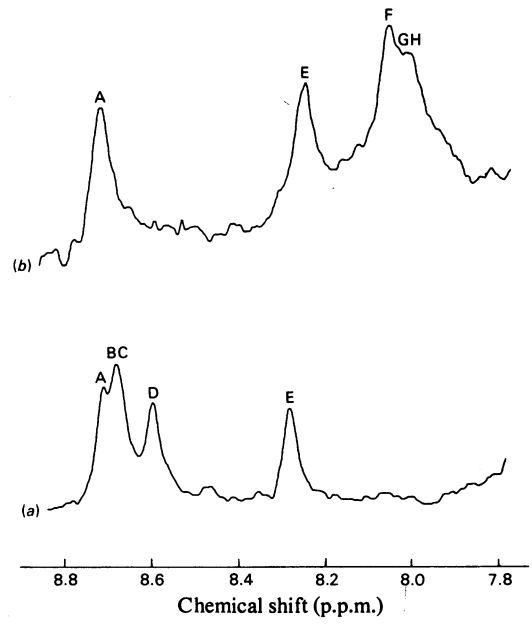


Fig. 2. *Histidine C-2 proton resonances of deuterium-exchanged β-lactamase II*

Spectra were collected after the amide protons of β-lactamase II had been exchanged for deuterium atoms by the method described in the Materials and Methods section. The enzyme concentration was 3.8mM. (*a*) Mono-zinc enzyme pH* 7.18. Relative peak intensities were E = 1.0, A, B, C and D = 3.8. (*b*) Apoenzyme, pH* 7.14. Relative peak intensities were E = 1.0, A = 0.9, F, G, H = 3.1.

^2H_2O to replace virtually all the amide protons by deuterium. Resonances in the region 7.8–8.8p.p.m. (Fig. 2*a*) are attributed to the C-2 protons of histidine residues, and the calculation of relative peak areas is feasible. If the area of the histidine resonance E at 8.28p.p.m. is taken as 1, the lower-field peaks (A, B, C, D) have an area of 3.8, indicating a total of five histidine residues. Amino acid analyses suggest β-lactamase II has five (Davies *et al.*, 1974) or six (Thatcher, 1975*b*) histidine residues.

The spectrum of the apoenzyme (prepared as described in the Materials and Methods section) is also simplified after deuterium exchange (Fig. 2*b*). Again, if the area of the histidine resonance at 8.24p.p.m. is taken as 1, the total area of the peaks visible in the spectrum between 7.8 and 8.8p.p.m. is 5.

pH titrations of histidine residues

Comparison of the spectra of the mono-zinc enzyme over the pH* range 6.0–9.0 revealed a single titrating residue, histidine E (Fig. 3). The experimental

values of chemical shifts for this resonance closely approximate to the theoretical curve for a group with pK_a*7.10 (Fig. 3, Table 1). The remaining resonances (A, B, C and D) remain unchanged throughout this pH range.

On titrating the mono-zinc enzyme between pH*6.0 and pH*4.7 small shifts are observed for resonances B+C and D (Fig. 3). Resonance B+C splits into two components (B and C), one of which shows a downfield and the other an upfield shift. Resonance D shifts upfield as one peak. The shift of all three resonances may be fitted to the theoretical curve of a group with pK_a* 5.15, and span 0.11, 0.13 and 0.21 p.p.m. respectively. Although all three resonances are assigned to histidine residues the group of pK_a* 5.15 does not have the characteristics of a histidine residue and so is not included in Table 1 (see the Discussion section).

Titration of the apoenzyme revealed a more complex situation. The aromatic region of the convolution difference spectrum of the apoenzyme at pH*5.23 is shown in Fig. 4. Five peaks (labelled A, E, F, G and H) attributed to the C-2 protons of the five histidine residues can be clearly seen. When the pH is raised, four of these histidine residues (E, F, G and H) titrate (Fig. 5), but the fifth (A) does not. The experimental values of the chemical shifts for resonance E closely approximate to the theoretical curve for a group with pK_a*7.02 (Fig. 5, Table 1). The experimental values of the chemical shifts for the remaining three resonances (F, G and H) approximate less closely to the theoretical curves for groups with pK_a* values of 6.68, 7.05 and 6.89 respectively.

A correlation between resonances in the spectra of the apoenzyme and the mono-zinc enzyme can be deduced from a comparison of Figs. 3 and 5. The correlation is implied in the labelling of resonances A and E in Figs. 2–5, and the group of resonances B, C and D of the mono-zinc enzyme correspond to the group of resonances F, G and H of the apoenzyme.

Exchange of histidine C-2 protons

Further characterization of histidine residues was obtained by incubation of enzyme samples in ^2H_2O at pH*8.05 and 37°C for 11 days. Spectra of the

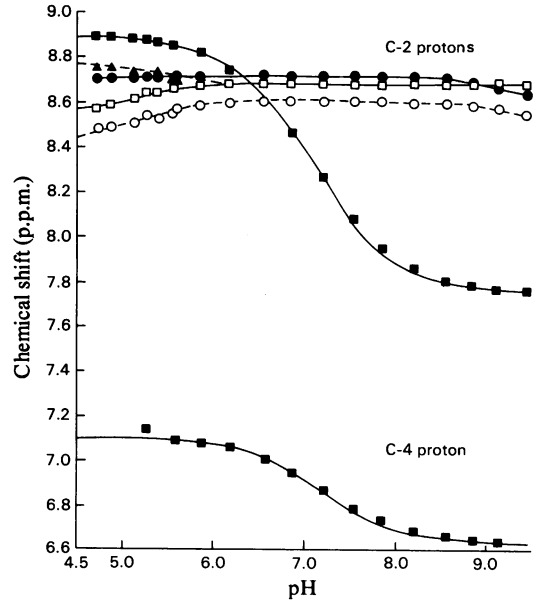


Fig. 3. *Titration curves of the C-2 and C-4 protons of the histidine residues of mono-zinc β-lactamase II*
The experimental values of chemical shift are shown by symbols, and the lines are theoretical curves calculated from the values of pK_a* and span listed in Table 1. The shifts observed below pH*6.0 for resonances B, C and D approximate to theoretical curves for a group of pK_a*5.15, and span 0.11, 0.13 and 0.21 p.p.m. respectively. Histidine residues: A (●); B (▲); C (□); D (○); E (■).

Table 1. *Properties of the histidine residues of β-lactamase II*
The values for pK_a* and span (the total change in chemical shift on protonation) of the C-2 and C-4 protons were obtained from direct linear plots (see the Materials and Methods section) of the experimental data. A resonance whose position did not change over the pH* range 6.0–9.0 is shown as NT (non-titrating). Approximate values for the half-lives for the exchange of the C-2 proton with solvent deuterium were determined at 37°C and pH*8.05. At this pH* resonances F, G and H are coincident.

Mono-zinc enzyme					Apoenzyme				
		Span (p.p.m.)		Approximate half-life			Span (p.p.m.)		Approximate half-life
Histidine	pK_a*	C-2 proton	C-4 proton	(days)	Histidine	pK_a*	C-2 proton	C-4 proton	(days)
A	NT	—	—	1	A	NT	—	—	1
B	NT	—	—	⩾11	F	6.68	1.15	0.46	3
C	NT	—	—	⩾11	G	7.05	1.01	0.54	3
D	NT	—	—	11	H	6.89	0.75	0.59	3
E	7.10	1.13	0.47	3	E	7.02	1.11	0.50	3

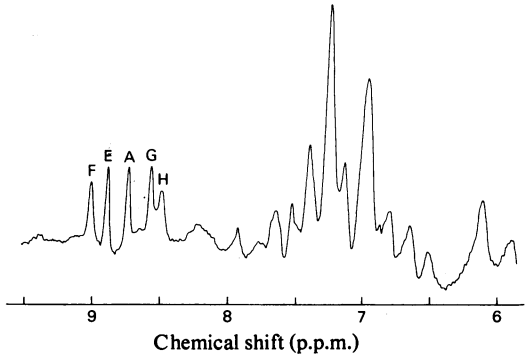


Fig. 4. *Convolution difference spectrum of apo-(β-lactamase II)*
The enzyme (5.3mM) was in 10mM-EDTA/20mM-succinate/20mM-triethanolamine/1M-NaCl, pH*5.23. Peaks A, E, F, G and H are assigned to the C-2 protons of five histidine residues.

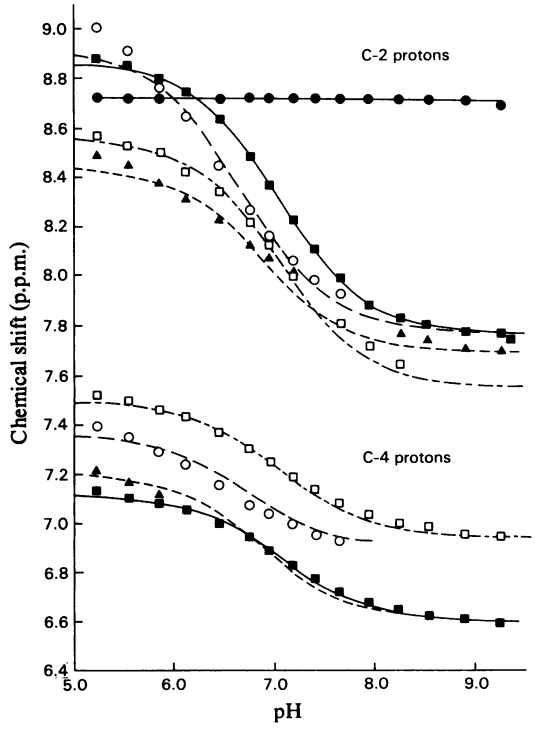


Fig. 5. *Titration curves of the C-2 and C-4 protons of the histidine residues of apo-(β-lactamase II)*
The experimental values of chemical shift are shown by symbols, and the lines are theoretical curves calculated from the values of pK_a* and span listed in Table 1. Histidine residues: A(●); E(■); F(○); G(□); H(▲).

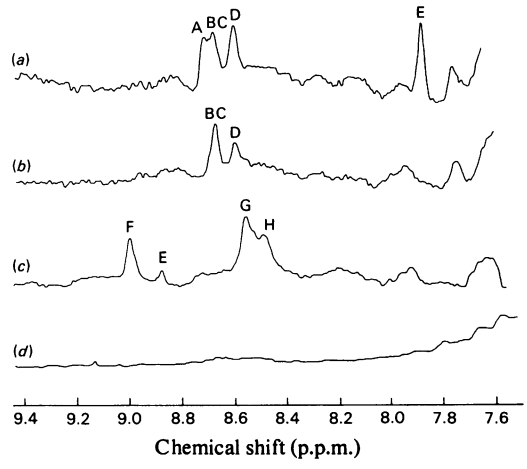


Fig. 6. *Deuterium exchange of histidine C-2 protons of β-lactamase II*
The enzyme (initially 4.0mM) was incubated in 20mM-succinate/20mM-triethanolamine/1M-NaCl in ^2H_2O, pH*8.05 at 37°C. Spectra were collected at 26.5°C. (*a*), (*b*) Convolution difference spectrum of mono-zinc enzyme at 0 and 11 days respectively, pH*8.05. (*c*) Convolution difference spectrum of apoenzyme prepared from sample (*b*), measured at pH*5.23 where the resonances are well resolved (see Fig. 4). (*d*) Spectrum of apoenzyme at 9 days, pH*8.05. Comparison of spectra (*c*) and (*d*) reveals that the presence of one equivalent of Zn(II) greatly retards deuterium exchange of the C-2 protons of histidine residues F, G and H.

mono-zinc enzyme, after this treatment, showed that resonances A and E had decreased in intensity by over 90%, owing to proton exchange with solvent deuterium. Resonances (B+C) and D were still present; the former had undergone no detectable loss of intensity, whereas resonance D had apparently lost about 45% of its original intensity (Figs. 6*a* and 6*b*). Some or all of this loss may be due to exchange of underlying resonances of amide protons. Dialysis against EDTA caused resonances B+C and D to be replaced by resonances in a position characteristic of resonances F, G, H in the apoenzyme (Fig. 6*c*). (A spectrum of the apoenzyme at the same pH but before proton exchange is shown in Fig. 4.) In contrast, the apoenzyme spectrum revealed the complete loss of all histidine C-2 proton resonances and all amide NH resonances between 8 and 11 p.p.m. within 9 days of incubation (Fig. 6*d*). The loss of the residual amide NH resonances in the mono-zinc enzyme was much less marked. Approximate half-lives for histidine C-2 proton exchange are given in Table 1.

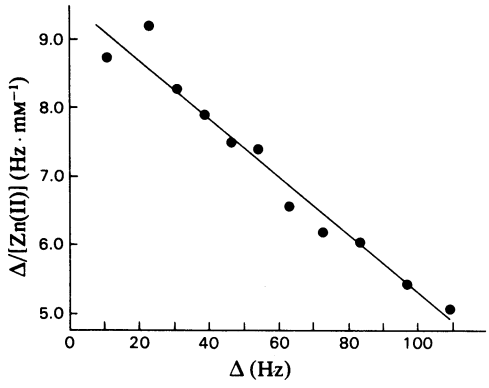


Fig. 7. *Scatchard plot for the binding of further Zn(II) to mono-zinc β-lactamase II*

The points show the change in chemical shift (Δ) observed for the C-2 proton resonance of histidine E on adding further Zn(II) to mono-zinc β-lactamase II (4.8mM in 20mM-succinate, 20mM-triethanolamine, 1M-NaCl, pH* 5.72). The line was drawn with a dissociation constant of 24mM and a maximum upfield shift on saturation of 225Hz (0.83p.p.m.), these values being obtained from a direct linear plot of the experimental data.

Zn(II) titrations of the first Zn(II)-binding site

Gradual addition of the first equivalent of Zn(II) to the apoenzyme at pH* 5.3 (where the resonances are well resolved) indicates that there is a progressive decrease of intensity at the position of the C-2 resonances of histidines F, G and H in the apoenzyme and an equivalent increase of intensity at the positions of resonances B+C and D in the mono-zinc enzyme. Thus the first Zn(II) ion bound by β-lactamase II is in slow exchange with free Zn(II). Additional changes observed include a small downfield shift for histidine E, and an apparent sharpening of resonances in a number of regions of the spectrum, most noticeable in the vicinity of 0p.p.m. These changes appear complete between 0.8 and 1.0mol of Zn(II)/mol of enzyme.

Zn(II) titrations of the second Zn(II)-binding site

Addition of further Zn(II) to mono-zinc β-lactamase II affects the position of histidine resonance E (Fig. 7). The continuous nature of the upfield shift, from the position of this histidine resonance at pH* 5.7, observed with increasing [Zn(II)] indicates that bound Zn(II) is in fast exchange with free Zn(II). A direct linear plot (Eisenthal & Cornish-Bowden, 1974), of shift against total [Zn(II)] yields a dissociation constant of 24mM and a maximum upfield shift on saturation of this zinc site of about 0.83p.p.m. at this pH*. (These values were obtained on the assumption that the free zinc concentration is close to the total zinc concentration.)

Discussion

Relative-intensity measurements have suggested the observation of resonances corresponding to five histidine residues in the protein. It should be noted that this represents a lower limit for the total number of histidine residues present, in that it is possible that not all of them may yield readily identifiable resonances (see, e.g., Browne *et al.*, 1976).

On adding one equivalent of Zn(II) to the apoenzyme resonances F, G and H are shifted downfield from their unprotonated positions and no longer titrate (Figs. 3 and 5). The lability of the C-2 protons of these residues is also observed to decrease markedly on zinc binding (Fig. 6); this may be a general method for identification of histidine residues acting as metal ligands. The changes in the behaviour of these three histidine residues strongly suggest that they serve as ligands to the first zinc. Hence the first metal-binding site in β-lactamase II probably consists in part of three histidine ligands, as with carbonic anhydrase (Kannan *et al.*, 1971); in addition there is evidence from chemical modification and spectroscopic studies (Davies & Abraham, 1974) that the thiol group of the single cysteine residue may also be involved.

The change in chemical shift for histidine E on binding the first zinc ion, and other changes throughout the n.m.r. spectrum, suggest that a conformational change accompanies the binding process. Circular-dichroism measurements (Davies & Abraham, 1974) have previously indicated a change in conformation on zinc binding.

The n.m.r. spectrum of the mono-zinc enzyme (Fig. 1) is also notable for the small number of resonances from amide protons of the polypeptide backbone. This observation, which is consistent with the results of hydrogen-exchange experiments (Kiener, 1976; Kiener & Waley, 1977), and with the ready digestibility of the native mono-zinc enzyme by trypsin at pH8.0 (G. Baldwin, unpublished work), suggests that the enzyme possesses unusual conformational motility despite its heat stability.

Some anomalies are observed in the titration behaviour of histidine resonances F, G and H in the apoenzyme. The shifts of these resonances cannot be precisely fitted to theoretical titration curves (Fig. 5). Since these are the resonances assigned to the zinc ligand histidine residues, which may remain close to each other on removal of zinc, one possible explanation is that the three ionizations are not independent. Similar anomalies have been observed in the titration behaviour of the ligand histidines of apo-(superoxide dismutase) (Cass *et al.*, 1977).

The titration behaviour at low pH of resonances B, C and D in the mono-zinc enzyme (corresponding

to resonances F, G and H in the apoenzyme) is not typical of freely titrating histidine residues. The observed chemical-shift span (Fig. 3) is small (0.1–0.2 p.p.m.), and in addition resonances C and D shift in the reverse direction to that expected for a freely titrating histidine residue. As suggested above, these resonances are probably the zinc ligands, and a possible explanation for their titration at low pH is the ionization of a nearby group (which is not a histidine residue) with pK_a^* 5.15, which is close to the zinc-binding site.

The large change in the chemical shift of the histidine E resonance on binding of a second zinc ion indicates that this residue is probably directly bound to zinc in this second site. The binding of the first zinc ion is obligatory for enzyme activity; the second zinc ion has a minor effect on activity, and even then only with some substrates (Davies & Abraham, 1974).

The remaining histidine resonance (A, Figs. 3 and 5 and Table 1) does not titrate over the pH* range 4.75–9.0. The value of its chemical shift suggests that it remains protonated up to pH*9, and it might therefore conceivably be hydrogen-bonded to a carboxylate ion. The relatively rapid exchange of the proton at C-2 of histidine A (Table 1) is also consistent with its remaining protonated at pH* 8.05 (Harris & Randall, 1965). Since the chemical shift and C-2 proton-exchange rate of histidine A are not affected by addition of Zn(II), it is most unlikely that it is a zinc ligand.

The main conclusions of the present work can be summarized as follows. (i) Three histidine residues are found as ligands in the first zinc-binding site of *B. cereus* β-lactamase II. (ii) A fourth histidine residue acts as a ligand in the second zinc-binding site. (iii) A fifth histidine residue is observed, which does not titrate between pH*4.75 and pH*9.0, but nevertheless is exposed to the solvent, as judged by C-2 proton–deuterium exchange.

Our results may have some bearing on catalysis. If the catalytically essential zinc ion is bound to four ligands (three histidine residues and the thiol group) and if it interacts directly (or via a water molecule) with the substrate, then the co-ordination geometry of the zinc is comparable with that suggested for alcohol dehydrogenase (Dworschack & Plapp, 1977) and carbonic anhydrase (Kannan *et al.*, 1977).

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