The ^1H Nuclear-Magnetic-Resonance Spectroscopy of Cobalt(II)- β -Lactamase II

Alphonse GALDES,* H. Allen O. HILL,* Graham S. BALDWIN,† Stephen G. WALEY† and Edward P. ABRAHAM†

*Inorganic Chemistry Laboratory and †Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 2QR, U.K.

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The ^1H n.m.r. spectra of β -lactamase II in the presence of Co(II) were studied. Analysis of the spectra suggests that Co(II) binds at the same two metal-binding sites as does Zn(II). The binding of Co(II) at the first site is much weaker than the binding of Zn(II) at this site, whereas the binding of Co(II) at the second site is tighter than the binding of Zn(II). The binding of Co(II) to the mono-zinc(II)-enzyme caused only one marked change in the spectrum, namely a decrease in the intensity of the resonances assigned to the C-2 and C-4 protons of one histidine residue (residue E). However, when the spectra of the apoenzyme and the Co(II)-enzyme were compared, there were many differences. A significant fraction of the protons in the whole molecule are affected by the binding of Co(II) at the first metal-ion-binding site (where the ligands are the enzyme's sole thiol group and three histidine residues). This may be because the first site is internal, or because of a difference in conformation between the apoenzyme and the mono-Co(II)-enzyme. The second site may be located on the surface of the molecule.

Bacillus cereus 569/H/9 produces two extracellular β -lactamases. One of these, β -lactamase II, appears to be unique among the known β -lactamases in its requirement of a metal ion for activity (Sabath & Abraham, 1966). The highest activity has been obtained with the Zn(II)-enzyme, but the Co(II)-, Cd(II)- and Mn(II)-enzymes are also active (Davies & Abraham, 1974). Equilibrium dialysis and measurements of absorption spectra have indicated that the enzyme can bind two Zn(II) or Co(II) ions (Davies & Abraham, 1974; G. S. Baldwin, A. Galdes, H. A. O. Hill, S. G. Waley & E. P. Abraham, unpublished work). Studies of the ^1H n.m.r. spectra have shown that three histidine residues act as Zn(II) ligands at the first (tighter) zinc-binding site (dissociation constant about $1\ \mu\text{M}$), and that a fourth histidine residue acts as a Zn(II) ligand at the second zinc-binding site (dissociation constant about $20\ \text{mM}$) (Baldwin *et al.*, 1978, 1979). From an investigation of the absorption spectra of the Co(II)- and the Cd(II)-enzyme it has been suggested that a thiol group is also involved in metal-ion binding at the first metal-ion-binding site (Davies & Abraham, 1974).

The substitution of the paramagnetic Co(II) for the diamagnetic Zn(II) is a common procedure in

the study of metalloenzymes (Vallee, 1973). This substitution results in the introduction of a spectroscopic probe and can thus be considered as a mild, but very selective, chemical modification. When the metal-ion-binding is followed by ^1H n.m.r. spectroscopy, the substitution of Zn(II) by Co(II) is expected to result in broadening and large changes in chemical shift for nuclei in the vicinity of the Co(II) ion (Dwek, 1973; Hill *et al.*, 1976). In particular, protons on the metal-ion-binding ligands are expected to be broadened beyond detection (Hill *et al.*, 1973, 1976). In the present work the binding of Co(II) to β -lactamase II was investigated by ^1H n.m.r. spectroscopy. The spectra were used to provide further information about the nature of the two metal-ion-binding sites in this enzyme.

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 $5\ \text{mM-NH}_4\text{HCO}_3$ containing $15\ \mu\text{M-ZnSO}_4$. ^2HCl (99.0% ^2H) and $^2\text{H}_2\text{O}$ (99.8% ^2H) were from Ryvan Chemical Co. (Southampton, U.K.). All other chemicals were of

AnalaR grade (BDH Chemicals, Poole, Dorset, U.K.) and were freeze-dried from $^2\text{H}_2\text{O}$ before use.

Preparation of samples

The apoenzyme was prepared for n.m.r. experiments as previously described (Baldwin *et al.*, 1978).

^1H n.m.r. experiments

All spectra were obtained at a frequency of 270 MHz with a modified Bruker console and a 6.4 T superconducting magnet (Oxford Instruments). The experimental techniques used have been described (Baldwin *et al.*, 1978). Chemical shifts are reported relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate as internal standard.

Typically, samples for n.m.r. experiments contained 4.5 mM-enzyme, 20 mM-succinate, 20 mM-triethanolamine and 1 M-NaCl in 0.4 ml of $^2\text{H}_2\text{O}$. Spectra were collected at 28°C.

Results

The general features of the 270 MHz ^1H n.m.r. spectra of mono-Zn(II)- β -lactamase II and the corresponding apoenzyme have been previously reported (Baldwin *et al.*, 1978). In the present paper the same nomenclature used previously for the five histidine resonances observed in these spectra is adhered to. Briefly, resonance A corresponds to a non-titrating histidine resonance not involved in metal-ion binding, resonance E corresponds to a histidine resonance involved in the binding of Zn(II) at the second metal-binding site, and resonances F, G and H in the apoenzyme [called B, C and D in the mono-Zn(II)-enzyme] correspond to the histidine residues involved in metal-ion binding at the first (tighter) zinc-binding site.

Effect of addition of Co(II) on the n.m.r. spectrum of the mono-Zn(II)-enzyme

Gradual addition of Co(II) to mono-Zn(II)- β -

Fig. 1. Co(II) titration of mono-Zn(II)- β -lactamase II

The enzyme (4.9 mM) was in 20 mM-succinate/20 mM-triethanolamine/1 M-NaCl buffer, pH* 5.7. Further experimental details are given in the text. (a) Convolution difference spectrum of the mono-Zn(II)-enzyme before the addition of Co(II); (b) as (a) with 2.8 mol of added Co(II)/mol; (c) difference spectrum (a)-(b); this reveals that the addition of Co(II) results in the loss of the C-2 resonance of histidine residue E.

lactamase II at pH*5.7 (pH* is the uncorrected meter reading in $^2\text{H}_2\text{O}$) results in a progressive decrease of intensity for the resonances associated with the C-2 and the C-4 protons of histidine residue E. Thus the Co(II) bound by mono-Zn(II)- β -lactamase II is in slow exchange with free Co(II). The intensity loss of these resonances appears complete at 2.8 mol of Co(II) added/mol of enzyme (Fig. 1). At this Co(II)/enzyme ratio less than 10% of the Zn(II) is replaced from the first site by Co(II) (see below) (G. S. Baldwin, A. Galdes, H. A. O. Hill, S. G. Waley & E. P. Abraham, unpublished work). No other changes in the n.m.r. spectra are observed over this range of Co(II) concentrations. Given that over 80% of the intensity of the histidine resonances is lost on addition of 2.8 mol of Co(II)/mol of protein, an upper limit of 2.5 mM for the dissociation constant of Co(II) can be calculated.

Effect of the addition of Co(II) on the n.m.r. spectrum of apo-(β -lactamase II)

Gradual addition of Co(II) to the apoenzyme at pH*5.7 results in very many changes throughout the whole ^1H n.m.r. spectrum (Fig. 2). Many resonances are broadened and are shifted a considerable distance from their original chemical-shift positions. This is most readily apparent in the regions outside

the main spectral envelope. Thus in the apoenzyme [and in the Zn(II)-enzyme] there are no resonances upfield of 0 p.p.m., but in the presence of Co(II) at least ten distinct resonances occur in this region (Fig. 2). The highest-field and lowest-field resonances observed are at -8.9 p.p.m. and at 27.5 p.p.m. respectively (results not shown). The bound Co(II) is in slow exchange with free Co(II).

In the region 7.5–9 p.p.m. the addition of Co(II) to the apoenzyme results in a progressive decrease of intensity for all five histidine resonances (Fig. 3) and in the gradual appearance of two new resonances, labelled A' and E' in Fig. 3, at 8.49 and 8.43 p.p.m. respectively. Both these resonances are sharp and hence cannot be due to amide protons (which frequently occur in this region of the spectrum), but must be due to C-bonded protons.

At 1 mol of Co(II)/mol of enzyme the intensities of resonances A, E, F, G and H are approx. 4% of those in the spectrum of the original apoenzyme (Figs. 3a and 3b). Addition of 1.4 mol of Co(II)/mol of enzyme results in a decrease of intensity of resonance E'. The intensity loss for this resonance is complete at about 2.5 mol of Co(II)/mol of enzyme. At 2.5 mol of Co(II)/mol of enzyme, only resonance A' is still present in this region of the spectrum (Fig. 3d). Addition of further Co(II) beyond 2.5 mol of Co(II)/mol of enzyme does not result in any further

Fig. 2. 270 MHz ^1H n.m.r. convolution difference spectra of apo- and Co(II)- β -lactamase II

The enzyme (2.3 mM) was in 20 mM-succinate/1 M-NaCl buffer, pH*6.0, at 28°C. Further experimental details are given in the text. S and HO 2 H mark the positions of the succinate and the pulsed H $_2$ O resonances respectively. The resonance at 0 p.p.m. is from the internal standard. The vertical scale of the low-field region is expanded $\times 4$ in both spectra. (a) Apo- β -lactamase II; (b) +2 mol of Co(II)/mol; the vertical scale is expanded $\times 2$.

Fig. 3. Histidine C-2 proton resonances for β -lactamase II in the presence of various concentrations of Co(II). The enzyme (4.5 mM) was in 20 mM-succinate/1 M-NaCl buffer. Further experimental details are given in the text. The labels A–H refer to the C-2 proton resonances (see the text for details). (a) Convolution difference spectrum for the apoenzyme at pH* 5.70; (b) +0.96 mol of Co(II)/mol, pH* 5.74; (c) +1.36 mol of Co(II)/mol, pH* 6.06; (d) +2 mol of Co(II)/mol, pH* 5.75.

changes in the n.m.r. spectrum. Essentially the same behaviour for the histidine resonances is seen at other pH* values, although at lower pH* values the binding of Co(II) is weaker. At pH* 5.25, for example, little change is observed in the n.m.r. spectrum until the Co(II)/enzyme ratio exceeds 0.4 mol/mol.

Addition of 1 equiv. of Zn(II) to a Co(II)-enzyme solution, containing 2 mol of Co(II)/mol of enzyme, results in the disappearance of the resonances associated with the Co(II) enzyme, including resonance A' and the resonances present outside the main spectral envelope, and in the concomitant appearance of the resonances typical of the mono-Zn(II)-enzyme. The latter resonances include histidine resonances A, B, C and D. As expected, resonance E is absent from this spectrum, owing to the effect of the Co(II) present in the solution.

pH titration

Comparison of the spectra of Co(II)-enzyme solution containing 1 mol of Co(II)/mol of enzyme over the pH* range 5.3–8.4 (where resonances A, A', E, E', F, G and H are still all present) reveals five titrating peaks, these being the residual four apoenzyme histidine resonances E, F, G and H and resonance E'. The titration of the apoenzyme histidine residues is identical with that previously described (Baldwin *et al.*, 1978). The experimental values of the chemical shift for resonances E' approximate to the theoretical curve for a group with a pK_a^* of 7.01 and chemical-shift span of 1.0 p.p.m. (Fig. 4).

The pH-titration behaviour of resonance E' is that expected of a histidine C-2 proton (Markley, 1975a). The corresponding C-4 proton is also observed (Fig.

Fig. 4. Titration curve for the C-2 and C-4 protons of the histidine residues of Co(II)- β -lactamase II

The solution contained 1 mol of Co(II)/mol of enzyme. The experimental values of chemical shift are shown by symbols, and the line for residue E' is a theoretical curve calculated for a pK_a of 7.01 and a chemical-shift span of 1.0 p.p.m. for the C-2 proton and of 0.52 p.p.m. for the C-4 proton. The titration curves for the residual apoenzyme resonances are not shown. All the enzyme resonances, including histidine residue A', show a small upfield shift (maximally approx. 20 Hz) relative to the internal standard (see the Materials and Methods section) with increasing pH^* in the presence of Co(II). This is probably due to an effect of the Co(II) on the internal standard. ●, Histidine residues A'; ■, histidine residue E'; C-2 proton upper curve, C-4 proton lower curve.

4), at 6.70 p.p.m. at pH^* 5.7. This resonance can be assigned to histidine E. The loss of resonance E' on the addition of further Co(II) beyond 1.4 mol of Co(II)/mol of enzyme (see above) is consistent with this assignment. Both the C-2 and the C-4 resonances of this residue are shifted 0.37 p.p.m. upfield by the bound Co(II) from the chemical-shift position observed in the apoenzyme (see Baldwin *et al.*, 1978).

The remaining resonances, A and A', do not titrate over this pH^* range (Fig. 4).

Exchange with solvent deuterium

When a sample of Co(II)-enzyme containing 2 mol of Co(II)/mol of enzyme is incubated in 2H_2O at pH^* 8.07 and $37^\circ C$ there is a gradual decrease in the intensity of resonance A', owing to exchange with solvent deuterons (Fig. 5). The half-life for this exchange process is estimated to be 2–3 days. The only amino acid side-chain proton that is expected to be labile under these conditions is the histidyl C-2 proton (Markley, 1975b), and therefore resonance A' is assigned accordingly. Spectra of the apoenzyme prepared from the Co(II)-enzyme that had been incubated for 7 days in 2H_2O show a marked decrease in the intensity of histidine resonance A (Fig. 5), whereas the intensities of the resonances assigned to the other histidine residues are largely unaffected. Hence resonance A' in the Co(II)-enzyme is assigned to histidine A, which is thus shifted 0.18 p.p.m. by the bound Co(II) from its chemical-shift position in the apo- and mono-Zn(II)-enzymes (see Baldwin *et al.*, 1978).

Discussion

The results reported above suggest that Co(II) binds at the same two metal-ion-binding sites on β -lactamase II as does Zn(II). Thus the disappearance of the C-2 and C-4 proton resonances associated with histidine residue E on addition of Co(II) to the mono-Zn(II)-enzyme (Fig. 1), and the diminished lability of the C-2 proton of this residue towards deuterium exchange in the presence of Co(II) (Fig. 5), indicate that Co(II) binds at the same second metal-ion-binding site as does Zn(II) (Baldwin *et al.*, 1978). The estimated dissociation constant of Co(II) for the second metal-ion-binding site shows that Co(II) binds at least 10 times more tightly than does Zn(II) at this site.

In addition, the very selective effect of Co(II) on the intensity of the resonances of histidine residue E suggests that this residue is located on the surface of the molecule, with few neighbouring amino acid side chains. This conclusion is consistent with the freely titrating behaviour observed for histidine residue E in the mono-Zn(II)-enzyme (Baldwin *et al.*, 1978). The half-life of about 3 days for the exchange of the C-2 proton of this residue in the apo- and mono-Zn(II)-enzymes (Baldwin *et al.*, 1978) with solvent deuterium is also that expected for a solvent exposed histidine residue (Markley, 1975b).

The disappearance of the histidine resonances F, G and H in the apoenzyme on addition of Co(II) (Fig. 3) and the diminished lability of the C-2 protons of these residues towards deuterium exchange in the presence of Co(II) (Fig. 5) suggest that Co(II) also binds at the first metal-ion-binding site for Zn(II) (Baldwin *et al.*, 1978). The addition of Zn(II) to the Co(II)-enzyme causes the reappear-

Fig. 5. Deuterium exchange of histidine C-2 protons of di-Co(II)- β -lactamase II

The enzyme (initially 5mM) was incubated in 20mM-succinate/20mM-triethanolamine/1M-NaCl buffer in $^2\text{H}_2\text{O}$, pH* 8.07, at 37°C. Spectra were collected at 28°C. (a) and (b) Convolution difference spectra of Co(II)-enzyme containing 2 mol of Co(II)/mol of enzyme at 0 and 5 days respectively at pH* 8.07. (c) Convolution difference spectrum of apoenzyme prepared from the Co(II)-enzyme after 7 days of incubation, measured at pH* 5.27, where the histidine C-2 resonances are well resolved (see Baldwin *et al.*, 1978).

ance of these histidine resonances, consistent with the displacement of Co(II) by Zn(II) from the first metal-ion-binding site. The stoichiometric replacement of Co(II) by Zn(II) from the first metal-ion-binding site shows that Zn(II) binds much more tightly than does Co(II) at this site. The present work and previous ^1H n.m.r. studies (Baldwin *et al.*, 1978) suggest that three histidine residues also act as metal-ion-binding ligands at this site.

At pH* 5.7 the addition of 1.4 mol of Co(II)/mol of enzyme results in the loss of the histidine resonances associated with the first metal-ion-binding site (F, G and H; Fig. 3), but the histidine resonance associated with the second metal-ion-binding site (E') is still visible (Fig. 3). The n.m.r. results suggest that 80–90% of the Co(II) bound to the mono-Co(II)-enzyme is bound at the first metal-ion-binding site, and that therefore the Co(II)

binding to the β -lactamase II can be adequately described by two microscopic dissociation constants. The calculated microscopic dissociation constants for the binding of Co(II) are 0.14 and 2.52mM respectively (G. S. Baldwin, A. Galdes, H. A. O. Hill, S. G. Waley, & E. P. Abraham, unpublished work); these may be compared with the corresponding Zn(II) dissociation constants of 0.7 μM and of 24mM respectively (Baldwin *et al.*, 1978; G. S. Baldwin, A. Galdes, H. A. O. Hill, S. G. Waley & E. P. Abraham, unpublished work). The calculated dissociation constant for Co(II)-binding to the second metal-ion-binding site is consistent with the estimated dissociation constant for Co(II) binding to the second metal-ion-binding site in the mono-Zn(II)-enzyme (see the Results section).

The large number of shifted and/or broadened resonances in the ^1H n.m.r. spectra of Co(II)-

β -lactamase II can be attributed to Co(II) binding at the first metal-ion-binding site. [This conclusion is based on the assumption that binding of Co(II) to the second metal-ion-binding site of the apoenzyme gives rise to the same small shifts observed in the Co(II) binding to the mono-Zn(II)-enzyme.] This indicates that, when Co(II) is bound at the first metal-ion-binding sites, a significant fraction of the protons in the protein are affected, and this would be consistent with the site being 'internal'.

Histidine residue A(A') in the Co(II)-enzyme does not titrate between pH* 5.3 and pH* 8.4, but readily undergoes C-2 proton-deuterium exchange. This residue has these same properties in the apo- and the mono-Zn(II)-enzymes (Baldwin *et al.*, 1978).

The binding of the Co(II) at the first metal-ion-binding site causes an upfield shift for the resonances of histidine residues A and E of 0.18 and 0.37 p.p.m. respectively (Fig. 3). Shifts for other unassigned resonances are also evident (Fig. 2). These paramagnetic-ion-induced shifts are dependent on the distance of a given proton from the ion, on the angle between the vector connecting it to the metal ion and on the principal magnetic axis (Dwek, 1973; Lenkinski *et al.*, 1978). However, the extraction of this information requires detailed knowledge of the symmetry of the metal ion (Lenkinski *et al.*, 1978) and of the magnetic susceptibilities of the metal-ion-enzyme complex in the principal axis directions (Hill *et al.*, 1973). This information is not yet available for any Co(II)-substituted enzyme, and hence no definite conclusion about the distance of the affected protons from the metal ion can be arrived at on the basis of the observed shifts.

The main conclusions (based partly on unpublished work by G. S. Baldwin, A. Galdes, H. A. O. Hill, S. G. Waley & E. P. Abraham) are: (i) Co(II) binds at the same two metal-ion-binding sites of *B. cereus* β -lactamase II as does Zn(II); (ii) the binding of Co(II) at the first metal-ion-binding site is much weaker than the binding of Zn(II) at this site, the

corresponding site dissociation constants being 0.14 mM and 0.7 μ M respectively (pH 6.0, 1 M-NaCl); (iii) the binding of Co(II) at the second metal-ion-binding site is much tighter than the binding of Zn(II) at this site, the site dissociation constants being 2.5 and 24 mM respectively; (iv) the first metal-ion-binding-site ligands to Co(II) are a thiol group and three histidine residues, this site being internal and the co-ordination geometry being related to that of a trigonally distorted tetrahedron; (v) the second metal-ion-binding site ligands includes a histidine residue, located on the surface of the molecule, the co-ordination geometry being related to that of an octahedron.

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