NMR EVIDENCE FOR THE STRUCTURE OF THE COMPLEX BETWEEN PENICILLIN AND THE DD-CARBOXYPEPTIDASE OF *STREPTOMYCES* R61

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1. Introduction

Streptomyces strain R61 excretes a DD-carboxypeptidase-transpeptidase [1] which seems to be closely related to the corresponding membrane-bound enzyme and appears to be a good model for the study of the interaction between the enzyme, its substrates and penicillin [2,3].

The soluble DD-carboxypeptidase forms a stable, enzymatically-inactive complex with penicillin which slowly breaks down to regenerate active enzyme and two breakdown products of penicillin, *N*-acyl-glycine and *N*-formyl-D-penicillamine [4,5]. A similar degradation of penicillin has been observed with the membrane-bound D-alanine carboxypeptidase of *Bacillum stearothermophilus* [6,7]. The mechanism by which penicillin is degraded by these enzymes, which involves the cleavage of the C_5–C_6 bond as well as the amide bond of the β-lactam, is not yet known.

A useful first step towards understanding this reaction would obviously be to establish the structure of the penicillin part of the penicillin–enzyme complex. It has recently been shown, for both the *Streptomyces* R61 DD-carboxypeptidase [8] and the membrane-bound D-alanine carboxypeptidase of *Bacillus subtilis* [9], that radioactively-labelled benzylpenicillin is covalently attached to a peptide in which the only chemically likely candidate for covalent attachment is a serine residue. This suggests that a penicilloyl enzyme is formed, the penicilloyl group forming an ester linkage to a serine residue (rather than the thioester linkage proposed [10]). A recent study of deuterium incorporation during the reaction [11] showed that in the long-lived penicillin–enzyme complex the C_5–C_6 bond of the penicillin is still intact, and is not a double bond (nor does it have a double-bonded precursor).

We now report direct evidence from NMR spectroscopy that the penicillin complex of the *Streptomyces* R61 DD-carboxypeptidase is indeed a penicilloyl–enzyme. In addition we show that the penicilloyl group is able to epimerise while still attached to the enzyme.

2. Materials and methods

The sodium salt of benzylpenicillin (penicillin G) and guanidinium chloride were obtained from Sigma Chemical Co. and [^{14}C]penicillin G from the Radiochemical Centre. Penicilloic acid and α-methyl-D-penicilloate were synthesised as in [12]. The enzyme was isolated and purified as in [1].

Streptomyces R61 DD-carboxypeptidase, 3.2 mg (84 nmol) (twice lyophilised from D_2O to remove exchangeable protons) was dissolved in 250 μl D_2O containing 10 mM potassium phosphate (pH 6.85) to give a final enzyme conc. 0.34 mM. The penicillin complex was formed by addition of exactly 1 equiv. benzylpenicillin, containing sufficient [^{14}C]benzylpenicillin to give a final spec. act. 14.3 mCi/mmol.

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The solution was incubated at 20°C for 10 min to ensure complete complex formation, and was then lyophilised. The lyophilised material was dissolved in 250 μl D_2O containing 6 M guanidinium chloride (extensively exchanged with D_2O), giving final pH 5.9. This solution was kept at 10°C for 25 days, then for 10 days at 20°C and finally 10 days at 30°C.

^1H NMR spectra were obtained periodically throughout this incubation period. All spectra were obtained at 20°C by the Fourier transform method on a Bruker WH270 spectrometer. The water signal was saturated by a selective 0.5 s pulse immediately before the observation pulse. 8192 data points were used for a 4200 Hz spectral width, and 4000 transients were accumulated for each spectrum. Chemical shifts are given in ppm from internal dioxan.

At intervals during the incubation, 1 μl samples of the reaction mixture were removed, spotted onto Whatman 3 MM paper and subjected to high voltage electrophoresis (Gilson High Voltage Electrophoreter at 4 kV) for 15 min in pyridine/acetic acid/water (25:1:474), pH 6.5. Radioactive spots were detected using a Packard Radiochromatogram Scanner, and the proportion of penicillin–enzyme complex still intact estimated from the proportion of the radioactivity (measured as peak areas) which remained at the origin.

3. Results and discussion

The aromatic region of the ^1H NMR spectrum of the native R61 enzyme is compared with that of its penicillin complex in fig.1. A number of differences between the two spectra can be seen, of which the clearest is the downfield shift of one of the eight histidine C2-H resonances from 4.55 ppm to 4.60 ppm on complex formation. (A separate pH titration gave no evidence for a change in the pK of this residue on complex formation.) The complexity of the spectrum is such that no resonances from the penicillin can be clearly identified; even the crucial region around 1.5 ppm downfield of dioxan contains too many resonances from the protein. A difference spectrum between the complex formed with benzylpenicillin and 6-^2H-benzylpenicillin showed no indication of the H-6 resonance, which must presumably have been hidden under the water resonance. Such a position

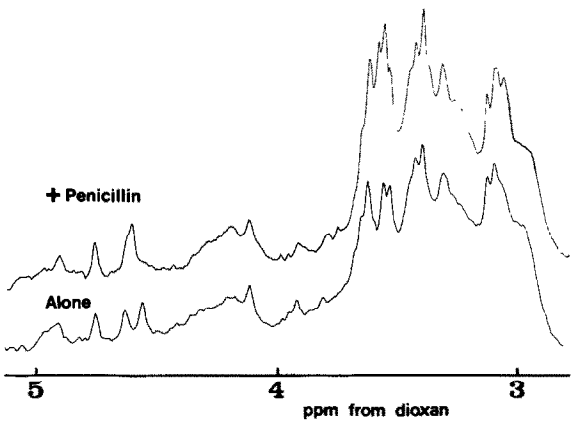

Fig.1. The aromatic region of the 270 MHz ^1H NMR spectrum of *Streptomyces* R61 DD-carboxypeptidase alone (bottom) and after addition of 1 molar equiv. of benzylpenicillin (top).

(consistent with the results obtained on the denatured complex; see below) would be quite distinct from the position of the corresponding resonance in benzylpenicillin. In order to simplify the region around 1.5 ppm and thus establish the structure of the penicillin in the complex, we examined the spectrum of the unfolded penicillin–enzyme complex, in 6 M guanidinium chloride. Under these conditions, the NMR spectrum is much simpler (as would be expected for a random-coil protein) and detection of penicillin resonances is thus facilitated. In addition, since secondary, environmental, shifts are absent, the structure of the penicillin in the complex can be more readily established by comparison of its resonances with those of model compounds.

The aromatic region of the spectrum of the complex in 6 M guanidinium chloride is shown in fig.2. In addition to the histidine C2-H resonance at 4.94 ppm and the (off-scale) resonances of aromatic and residual guanidinium protons in the centre of the spectrum, two small resonances at 1.47 ppm and 1.36 ppm can just be observed. Since no amino acid resonances would be expected to occur in this region for a random-coil protein, it seems likely that these resonances arise from the penicillin. Further evidence for this comes from the time dependence of the intensity of these resonances. Incubation of the penicillin–enzyme complex in 6 M guanidinium chloride leads to a slow breakdown of the complex.

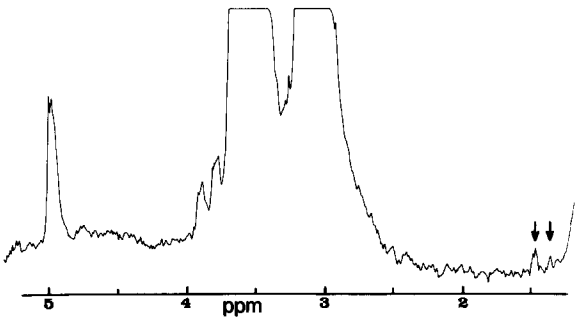

Fig.2. The aromatic region of the 270 MHz ^{1}H NMR spectrum of the complex between *Streptomyces* R61 DD-carboxypeptidase and benzylpenicillin in 6 M guanidinium chloride. The off-scale resonances in the centre of the spectrum are those of the bulk of the aromatic protons and of the residual guanidinium protons. The penicillin signals are arrowed.

Table 1 compares the decrease in intensity of the two resonances at 1.47 and 1.36 ppm with the decrease in the proportion of intact complex, measured electrophoretically. The close correspondence between the two sets of figures provides strong evidence that these two resonances do arise from the penicillin–enzyme complex. It is also apparent from table 1 that the sum of the areas of these two resonances corresponds to one proton in the complex, so that the complex must exist in two forms. These appear to interconvert slowly, their relative areas changing from 2:1 at the beginning of the incubation to 1:1, which seems to be the equilibrium value, after 14 days.

It is immediately clear that no non-covalently bound benzylpenicillin, which would become free benzylpenicillin on addition of 6 M guanidinium chloride, is present, since there is no sign of its characteristic H-5 resonance at 1.77 ppm. As a model of the proposed serine-linked penicilloyl–enzyme, we have examined α-methyl-D-penicilloate, in which the β-methylene of the serine residue is represented by a methyl group. In 6 M guanidinium chloride (pH 5.9) the H-5 and H-6 resonances of [5R,6R]-α-methyl-D-benzylpenicilloate appear at 1.42 ppm and 0.85 ppm, respectively. It is possible, therefore, that the resonance at 1.47 ppm seen in the complex is indeed that of the 5-proton of the penicilloyl–enzyme. Further evidence for this assignment comes from time dependence studies and from spin decoupling experiments.

On incubation in water [5R,6R]-α-methyl-D-benzylpenicilloate has been shown [13] to slowly epimerise to the [5S,6R] compound. The same process can be observed in 6 M guanidinium chloride, the [5S,6R] enantiomer having an H-5 resonance at 1.34 ppm (the H-6 resonance was obscured by the water signal). The close correspondence in chemical shift to the signal in the penicillin–enzyme complex at 1.32 ppm, together with the fact that the relative intensity of the latter peak increases with time,

Table 1
Time-dependence of the concentration of the penicillin–enzyme complex and of the intensities of the resonances at 1.47 ppm and 1.36 ppm

Incubation time (days)	Resonance intensity		(no. of H$^+$)[a] sum of (1) and (2)	Fraction of complex remaining[b]
	(1) 1.47 ppm	(2) 1.36 ppm		
0	0.52	0.26	0.78	0.78
2	0.51	0.24	0.75	0.77
3	0.48	0.24	0.72	[c]
6	0.43	0.28	0.68	[c]
12	0.35	0.29	0.64	0.70
14	0.31	0.30	0.61	[c]
21	[c]	[c]	[c]	0.62
26	0.20	0.20	0.40	0.44

[a] Measured relative to the 8-proton histidine C2-H resonance at 4.96 ppm
[b] Determined by electrophoretic analysis of radiolabelled complex: see section 2
[c] Not determined

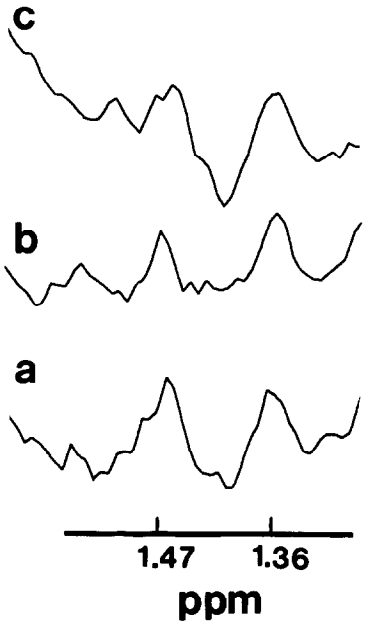

Fig.3. The penicillin resonances of the *Streptomyces* R61 DD-carboxypeptidase–benzylpenicillin complex after incubation in 6 M guanidinium chloride for several days. (a) No irradiation. (b) Irradiation at 0.89 ppm. (c) Irradiation at 0.79 ppm (control).

supports the assignment to a penicilloyl–enzyme and suggests that the two forms of the complex are the [5R,6R] and [5S,6R] enantiomers. A further characteristic spectral difference between these 2 enantiomers is the magnitude of the spin–spin coupling constant between the 5- and 6-protons, which is 6 Hz in [5R,6R]- and 2 Hz in [5S,6R]-α-methyl-D-benzylpenicilloate. Figure 3 shows the two penicillin resonances of the complex, at a time when their areas have become roughly equal. Although, because of the relatively low enzyme concentration, the signal-to-noise ratio is poor, a clear indication of doublet structure can be seen on the lower-field signal, which should have a splitting of 6 Hz, but not on the higher-field signal, whose smaller splitting is only of the same order as the linewidth. Double resonance experiments (fig.3) show that there is a marked sharpening of the resonance at 1.47 ppm on irradiation at 0.89 ppm, due to collapse of the doublet splitting (compare fig.3b,c). The H-6 resonance must thus be at 0.89 ppm, very close to the position of the corresponding resonance in [5R,6R]-α-methyl-D-penicilloate; direct observation of this resonance in the complex is not possible, since it is obscured by the water resonance and by α-proton signals.

Although the evidence relates only to the denatured complex, and covalent changes accompanying denaturation cannot be completely ruled out, the shifts, coupling constants, time-dependence and double resonance behaviour of the 1.47 and 1.36 ppm resonances of the penicillin complex of *Streptomyces* R61 DD-carboxypeptidase are all completely consistent with the proposal that the complex exists as a penicilloyl–enzyme.

Acknowledgements

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