Identification by n.m.r. spectroscopy of a stable intermediate structure in the unfolding of staphylococcal β -lactamase

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The unfolding of β -lactamase (penicillinase) from *Staphylococcus aureus* by guanidinium chloride was followed by using n.m.r. spectroscopy. On the basis of the observation of resonances corresponding to histidine, tyrosine and other amino acid side chains, the existence of a stable partially folded species was demonstrated. These experiments provide detailed characterization of the intermediate that confirms and extends previous characterization by absorption and c.d. spectroscopy and by flow properties. In addition, they show that residues in the *N*-terminal third of the molecule are affected by the native-to-intermediate transition. Persistent non-equivalence of the two imidazole C2 proton resonances at high guanidinium chloride concentrations is discussed in terms of local sequence effects on the chemical shift.

β -Lactamase (penicillinase) from *Staphylococcus aureus* has been studied as an interesting model for protein folding (Robson & Pain, 1973; Adams *et al.*, 1980). It is a monomeric single-polypeptide protein of M_r 28000, with no disulphide bonds and no tryptophan residues (Ambler, 1975). It provides the clearest example of a globular protein that can be partially denatured by GuCl to a thermodynamically stable species, state H, whose conformation is intermediate between the native and unfolded states N and U (Robson & Pain, 1976*a,b*). State H, which is stable in 0.8M-GuCl, is characterized by its 13 tyrosine residues being fully exposed to solvent and lacking the degree of environmental asymmetry exhibited in state N. It is considerably expanded ($f/f_0=1.9$) compared with state N, but without appreciable decrease in helix and β -sheet secondary structure as judged by far-u.v. c.d. (Carrey & Pain, 1978). State U, present above 2M-GuCl, has been characterized by having a far-u.v. c.d. spectrum characteristic of a random coil or a periodic structure.

State H is in reversible equilibrium with states N and U, the rates of interconversion being slow ($t_1 > 1$ min) and fast ($t_1 < 1$ s) respectively, and the transitions behave thermodynamically as two state

transitions. State H differs from both states N and U by a free-energy difference of approx. 10 kJ/mol.

A further transient state, I, has been shown to accumulate kinetically by urea-gradient/polyacrylamide-gel electrophoresis (Creighton & Pain, 1980). This is a compact condensed state in fast equilibrium with state H but interconverting only slowly with state N. The slow recovery of native tyrosine environment and of enzyme activity thus corresponds to the transition from state I to state N.

On the basis of the above and of immunological results, a model has been proposed in which β -lactamase is folded into three closely packed domains that separate, without themselves unfolding, to give state H (Carrey & Pain, 1977; Adams *et al.*, 1980).

To characterize further the folding of β -lactamase we have studied the ^1H -n.m.r. spectrum of the protein as a function of GuCl concentration. The enzyme contains only two histidine residues, at positions 16 and 63 (Ambler, 1975), which provide 'markers' for the *N*-terminal third of the molecule. The results obtained support and extend those obtained from the previous investigations.

Materials and methods

Materials

β -Lactamase was isolated from fermentation supernatants of *Staphylococcus aureus* PC1 and

Abbreviation used: GuCl, guanidinium chloride.

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purified as described by Robson & Pain (1976a). It was shown to be homogeneous by gel filtration and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Trypsin (type XI, treated with diphenylcarbamoyl chloride) was obtained from Sigma Chemical Co., and *S. aureus* V8 proteinase from Miles Laboratories. Proteolytic digestion of β -lactamase was carried out in ammonium acetate, pH 8.7, as described by Ambler (1975) and checked by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. GuCl was AristaR from BDH Chemicals.

N.m.r. spectroscopy

N.m.r. spectra were recorded with the use of a Bruker WH270 spectrometer. $\text{Gu}[\text{}^2\text{H}]\text{Cl}$ was produced by extensive exchange of $\text{Gu}[\text{}^1\text{H}]\text{Cl}$ with ${}^2\text{H}_2\text{O}$. Labile protons and water in penicillinase were exchanged with ${}^2\text{H}_2\text{O}$ by freeze-drying. In a typical experiment 0.4 ml of a solution of β -lactamase (25 mg/ml) in 10 mM-sodium phosphate buffer, pH 7.0, containing 90 mM-NaCl and 1 mM-EDTA was placed in an n.m.r. tube, and 1 μl of 1,4-dioxan (10%, v/v, in ${}^2\text{H}_2\text{O}$) was added as internal reference. A spectrum was measured, after which 6 M- $\text{Gu}[\text{}^2\text{H}]\text{Cl}$ in ${}^2\text{H}_2\text{O}$ was added to give the desired concentration of GuCl. A further spectrum was then measured after allowing the system to come to equilibrium. For the higher concentrations (2–6 M) of GuCl, either enzyme freeze-dried from 1 mM-phosphate buffer was dissolved directly in the appropriate solution of $\text{Gu}[\text{}^2\text{H}]\text{Cl}$ in ${}^2\text{H}_2\text{O}$ or portions of solid GuCl were added to the enzyme solution. There was no significant change in apparent pH value on addition of GuCl.

Typically 2000 transients were collected for each spectrum. In some experiments a delay of 5 s was inserted between pulses and the ${}^1\text{H}_2\text{O}$ resonance selectively saturated by irradiation for 0.5 s immediately before the main pulse. Spectra were recorded at 25°C unless otherwise stated. Chemical shifts are quoted with respect to dioxan (3.71 p.p.m. downfield from 4,4-dimethylsilapentane-1-sulphonate).

Results and discussion

Native β -lactamase

The aromatic region of the 270 MHz ${}^1\text{H}$ -n.m.r. spectrum of native β -lactamase is shown in Fig. 1(a). This part of the spectrum is dominated by four large peaks, D–G, arising from tyrosine and phenylalanine residues, the spectrum being too complex to allow detailed assignments of these peaks. Immediately to low field of these resonances is a shoulder, C, which may have contributions from phenylalanine protons. Still further downfield, two imidazole C2 proton resonances can be seen (A and

B), which correspond to the two histidine residues of the protein (His-13 and His-63). The peak at the higher field (B) has a chemical shift of 3.97 p.p.m., typical of a protein histidine residue exposed to the solvent (Wüthrich, 1976; Jardetsky & Roberts, 1981), and the other (A), at 4.58 p.p.m., suggests a histidine residue in a more fully protonated form than expected at pH 7, presumably owing to interaction with an anionic group. Underlying the histidine signals are broad resonances due to backbone amide protons.

The signals in the aliphatic region of the n.m.r. spectrum of the native enzyme are broad and complex (see Fig. 3a). The large peak at approx. –0.7 p.p.m. corresponds to lysine αCH_2 proton resonances, and that at –2.1 p.p.m. includes resonances from lysine β - and $\delta\text{-CH}_2$ protons (1, 2). The broad signal centred on –3 p.p.m. arises from side-chain methyl protons. Between –3.0 and –3.5 p.p.m. there are several broad shoulders corresponding to ‘high-field’ methyl resonances; these arise from methyl protons in close proximity to the faces of aromatic rings in the folded structure. The signal at –3.71 p.p.m. varies in intensity in different samples and persists at this high chemical-shift value even in 6 M-GuCl: it is almost certainly due to a silicone impurity in the sample (SiMe_3 protons have a chemical shift –3.7 p.p.m. from dioxan).

β -Lactamase in 0–0.8 M-GuCl

The addition of GuCl to β -lactamase in the concentration range up to 0.8 M-GuCl, where state H becomes the predominant species, induces marked changes in the aromatic region of the ${}^1\text{H}$ -n.m.r. spectrum (Fig. 1). The behaviour of the histidine C2 proton resonance is particularly interesting. Peak B, corresponding to the more exposed histidine residue, shifts to low field over this range of GuCl concentration, but does not change in intensity. On the other hand, peak A, corresponding to the C2 proton of the ‘abnormal’ histidine residue, progressively decreases in intensity and shifts slightly to lower field, as demonstrated in Figs. 1 and 2. At 0.8 M-GuCl it has completely disappeared. As peak A disappears, a new histidine C2 proton signal, labelled A' in Fig. 1, appears at higher field, this time with a resonance position close to peak B. At a concentration of approx. 0.6 M-GuCl peaks A and A' have similar intensities and are each about 50% of the size of peak B. The coexistence of peaks A and A' with complementary intensities indicates that they arise from the same histidine residue in two different conformers of the protein that are in slow exchange with each other. One species has a buried histidine residue and an exposed histidine residue, whereas the other has both histidine residues exposed to the solvent to a similar extent. The differences between the two forms of the protein

Fig. 1. Aromatic region of the 270 MHz ^1H -n.m.r. spectra of staphylococcal β -lactamase at 0–4.1 M-GuCl at 20°C (a) Enzyme alone (25 mg/ml in 10 mM-phosphate buffer, pH 7); (b) in 0.42 M-GuCl; (c) in 0.73 M-GuCl; (d) in 0.81 M-GuCl; (e) in 2.7 M-GuCl; (f) in 4.1 M-GuCl. Spectra are not all on same intensity scale (e and f different). Samples for (e) and (f) were prepared by adding solid GuCl to the samples. Chemical shifts were measured relative to 1,4-dioxan as an internal reference.

must be such that interconversion involves a substantial activation energy: the observation of two separate signals ($\nu_A - \nu_{A'} = 140\text{ Hz}$) indicates that the rate of interconversion is $< 70\text{ s}^{-1}$. If the differences were due to structural 'flickering' between two energetically similar states, the exchange between the species would be expected to be rapid and only one histidine resonance A, intermediate in position between 4.1 and 4.65 p.p.m., would be seen.

In the baseline around the C2 proton resonances can be seen several broad resonances from amide protons, which exchange only slowly in the native β -lactamase. These disappear much more rapidly in the presence of 0.8 M-GuCl, indicating that amide proton exchange occurs more rapidly in state H than in state N.

In 0.8 M-GuCl two of the major aromatic bands (C and F in Fig. 1) have completely disappeared, whereas peak E has considerably increased in

Fig. 2. Dependence of ^1H chemical shifts of β -lactamase histidine C2 proton resonances on GuCl concentration. Chemical shifts were measured relative to 1,4-dioxan as an internal reference. ●, Signal A (see Fig. 1); ■, signal B; ▲, signal A'.

intensity. This signal is at the resonance frequency expected for C2' and C6' protons in solvent-accessible tyrosine residues. The higher-field doublet of the exposed tyrosine is presumably hidden by the peak contributed by $\text{Gu}^1\text{H}\text{Cl}$, which at higher denaturant concentrations completely obscures the region around peak G. Thus the aromatic region of the spectrum shows that several tyrosine residues have been transferred to an unstructured environment on going from state N to state H. It should be noted that u.v. difference spectroscopy has shown that tyrosine residues in native penicillinase are 50% exposed (Robson & Pain, 1976a).

The absence of resolvable fine structure on the aromatic resonances indicates that not all the aromatic residues are magnetically equivalent, implying that several of them are still in structured parts of the protein. Near-u.v. c.d. and difference spectra indicate that these non-exposed tyrosine residues cannot be in an optically asymmetric environment and that their environment must be, on average, polar (Robson & Pain, 1976a; Carrey & Pain, 1978).

Fig. 3. Aliphatic region of the 270 MHz ^1H -n.m.r. spectra of staphylococcal β -lactamase at 0–4.1 M-GuCl at 20°C (a) Enzyme alone (25 mg/ml in 10 mM-phosphate buffer, pH 7); (b) in 0.81 M-GuCl; (c) in 2.0 M-GuCl; (d) in 2.7 M-GuCl; (e) in 4.1 M-GuCl. Samples (d) and (e) were prepared on a separate sample of enzyme and to which solid GuCl was added to the solutions. This contained a higher content of silicone impurity.

In the aliphatic region of the ^1H -n.m.r. spectrum, although there is some simplification on going to 0.8 M-GuCl (Fig. 3b), there is clear evidence that considerable structure remains in state H; this is entirely consistent with the results from c.d. and viscosity measurements. In this range, the high-field methyl signals (–3.5 to –3.7 p.p.m. in the native-enzyme spectrum) disappear and the intense resonance corresponding to methyl protons (approx. –2.9 p.p.m.) sharpens considerably. Small signals J and I, not apparent in the native-enzyme spectrum, are now resolved: J (approx. –1.71 p.p.m.) has a chemical shift corresponding to that of methionine methyl protons in unfolded proteins.

Penicillinase in 0.8–2.7 M-GuCl

The u.v. c.d. data indicate that at 2 M-GuCl the regular secondary structure of state H is lost as the protein goes to state U. These changes are reflected in the ^1H -n.m.r.-spectral changes in this range of denaturant concentration. There is additional sharpening of the peaks in the aromatic region (see

Fig. 1e), and in the aliphatic region of the spectrum the methyl proton resonance at –2.9 p.p.m. narrows by a further 20% (Fig. 3c). A second methionine methyl resonance (I') appears near to the chemical-shift positions for methionine in unfolded protein. This is accompanied by the disappearance of the sharp signal I observed in the spectrum from the 0.8 M-GuCl solution (Fig. 3b), and it is possible that this arises from a methionine residue that has undergone a change in environment at 2 M-GuCl such that it is now more similar to a methionine residue in denatured protein, giving rise to I' (Fig. 3c).

In this range the two histidine C2 proton resonances show only minor changes in chemical shifts: at 2 M-GuCl they accidentally have the same chemical shifts, but at higher GuCl concentration (2.7 M; Fig. 1e) separate signals are observed once more, with signal B now being the higher-field signal. An additional small signal at 4.71 p.p.m. was occasionally observed in some samples (Fig. 1e). This varied in intensity in the different samples,

strongly suggesting that it arose from an impurity; it corresponded in chemical shift to the formate proton.

β -Lactamase in 2.7–6 M-GuCl

In this range very little change in the spectra was noted (compare Figs. 1e and 1f and Figs. 3d and 3e). Even at 6 M-GuCl the two histidine C2 proton signals remained non-equivalent. In an attempt to bring the histidine residues into a single unfolded environment the temperature of the protein solution in 6 M-GuCl was raised. Even after being heated to 80°C, separate signals A and B of equal intensity persisted in the spectrum. The existence of these two separate signals at concentrations above 2 M-GuCl suggests either that there is residual folded conformation under these conditions or that there are particular influences of neighbouring residues on the chemical shift of one or both of the histidine residues. In order to test the latter hypothesis, β -lactamase was cleaved by trypsin to give peptides Tyr-10...His-13...Lys-22 and Val-62...His-63...Lys-66 and by staphylococcal proteinase to give peptides Lys-8...His-13...Tyr-17 and Lys-52...His-63...Glu-77.

The ^1H -n.m.r. spectrum of the unfractionated trypsin digest showed only a single broad signal ($\Delta\nu_{\frac{1}{2}} = 12\text{ Hz}$) for the histidine C2 protons, corresponding in chemical shift to fully exposed histidine residues in a random-coil polypeptide chain. The unfractionated staphylococcal-proteinase digest gave a very broad signal extending over 30 Hz. This result suggests the possibility that local chemical-shift effects in a random-coil polypeptide could be responsible for the persistent non-equivalence of the two imidazole C2 proton resonances. For example, the time-average proximity of a charged group (e.g. from a lysine side chain) might affect the pK and hence the chemical shift of a histidine residue.

Conclusions

The n.m.r. spectra obtained for β -lactamase in 0.8 M-GuCl demonstrate the stable existence in this solvent of a species that has a conformation

intermediate in complexity between the native state N and the fully unfolded state. The behaviour of signal A, originating from one of the two histidine residues, shows that there is a slow equilibrium between two conformers that differ markedly in the environment of this residue. The conformers differ also in the environments of tyrosine and other amino acid side chains. These experiments provide detailed confirmation of the existence of state H, as previously characterized by absorption and c.d. spectroscopy and by flow properties. In addition, they show that residues in the N-terminal third of the molecule are affected by the state-N-to-state-H transition. State H must differ from state N in the solvent exposure of one of the two histidine side chains, in addition to the corresponding difference in some of the tyrosine residues detected by optical spectroscopy.

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