pK_a measurements from nuclear magnetic resonance of tyrosine-150 in class C β-lactamase

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^{13}C-NMR spectroscopy was used to estimate the pK_a values for the Tyr^{150} (Y150) residue in wild-type and mutant class C β-lactamases. The tyrosine residues of the wild-type and mutant lactamases were replaced with ^{13}C-labelled L-tyrosine ([*phenol*-4-^{13}C]tyrosine) in order to observe the tyrosine residues selectively. Spectra of the wild-type and K67C mutant (Lys^{67} → Cys) enzyme were compared with the Y150C mutant lactamase spectra to identify the signal originating from Tyr^{150}. Titration experiments showed that the chemical shift of the Tyr^{150} resonance in the wild-type enzyme is almost invariant in a range of 0.1 p.p.m. up to pH 11 and showed that the pK_a of this residue is well above 11 in the substrate-free form. According to solvent accessibility calculations on X-ray-derived structures, the phenolic oxygen of Tyr^{150}, which is near the amino groups of Lys^{315} and Lys^{67}, appears to have low solvent accessibility. These results suggest that, in the native enzyme, Tyr^{150} in class C β-lactamase of *Citrobacter freundii* GN346 is protonated and that when Tyr^{150} loses a proton, a proton from Lys^{67} would replace it. Consequently, Tyr^{150} would be protonated during the entire titration.

Key words: active-site mimics, buried surface, cephalosporinase, matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF), phenolate formation, solvent accessibility.

INTRODUCTION

Class C β-lactamases are serine β-lactamases that have cephalosporinase activity. Their amino acids have been sequenced, several X-ray crystal structures of the free enzyme and inhibitor-bound enzymes have been solved [1–7], and site-directed-mutagenesis experiments have identified residues important for catalysis [8–14]. Studies on class C β-lactamase have suggested that Tyr^{150} may be deprotonated at physiological pH and may act as the general acid–base involved in the enzyme reaction [1,2,6,15]. We have previously reported small molecules that mimic the active site of class C β-lactamases. Studies using these molecules suggested that the phenolic moiety mimicking Tyr^{150} is deprotonated near physiological pH [16,17]. On the other hand, the recent crystal structure of a S67A (Ser^{67} → Ala) mutant complexed with a cephalosporin derivative showed that the negatively charged carboxylate of the cephalosporin interacts with Tyr^{150} within hydrogen-bond distance, suggesting that Tyr^{150} is protonated [18]. In addition, the pK_a calculated by the Poisson–Boltzmann method for Tyr^{150} in *Enterobacter cloacae* P99 β-lactamase, which has 77% sequence homology with *C. freundii* GN346 β-lactamase, was 8.3 [19], suggesting that the pK_a of Tyr^{150} is considerably lowered, but the residue is protonated at physiological pH. Thus it is desirable to measure the pK_a of Tyr^{150} to know whether the phenolic moiety is protonated or not. In the present study we have used the actual enzyme and its mutant to directly assess the pK_a of Tyr^{150}.

An NMR titration method can be applied to observe the protonated states of particular residues in proteins. The measurement of ionization constants in proteins using NMR spectroscopy is well-established [20]. Here we describe estimation of pK_a values of Tyr^{150} side chain in the active site of wild-type and K67C mutant class C β-lactamase from *C. freundii* GN346.

MATERIALS AND METHODS

Materials

Epicurian Coli® BL21 (DE3) cells (competent cells available from Stratagene) was used as the host cell. pCFC-Plac-1, pCFC-Plac-Lys67Cys and pCFC-Plac-Tyr150Cys, prepared by inserting the wild-type, K67C and Y150C *C. freundii* GN346 β-lactamase genes respectively under control of the *lac* promoter in a pHSG298 vector [20a] were generously provided by Emeritus Professor Tetsuo Sawai (Division of Microbial Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan). L-[*phenol*-4-^{13}C]Tyrosine was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, U.S.A. Heart Infusion Agar was purchased from Eikenyaku (Tokyo, Japan), and was prepared as directed. Vitamin Mix (Gibco BRL) was dissolved as directed. DNase II (D4138) was purchased from Sigma. For cation exchange and gel filtration, 5 ml Hi-Trap SP cartridges and Superdex 75 (Amersham Pharmacia Biotech) were used respectively and chromatography was performed on an Amersham Pharmacia Biotech FPLC system. All other chemicals were purchased from Nacalai Tesque, Suita-shi, Osaka, Japan.

Transfection of vectors carrying the *C. freundii* GN346 β-lactamase gene into Epicurian Coli® BL21 (DE3) cells and cultivation of the transformed cells

Competent cells of Epicurian Coli® BL21 (DE3) were separately transfected with pCFC-Plac-1, pCFC-Plac-Lys67Cys and pCFC-Plac-Tyr150Cys as directed by Stratagene. The transformed cells

were spread on Heart Infusion Agar plates and were incubated at 37 °C overnight. A single colony was transferred to a modified M9 medium based on an amino-acid-specific labelling medium used by Markley and Kainosho [21] and was incubated at 37 °C with vigorous shaking. One litre of medium (pH 7) consisted of 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 1 ml of 100 mM $CaCl_2$, 1 ml of 1 M $MgSO_4$, 1 g of glucose, 3 ml of vitamin mix, 25 mg of nicotinic acid, 50 mg of adenine, 25 mg of thiamin, 25 mg of each of the 20 L-amino acids (where L-tyrosine was replaced with L-[*phenol*-4-^{13}C]tyrosine) and 50 mg of kanamycin. Upon isopropyl β-D-thiogalactoside ('IPTG') addition at an attenuance (D_{600}) of about 0.8 to induce lactamase production, the culture was incubated for another 16 h.

Purification of β-lactamases

Procedures used by Tsukamoto et al. [8] were modified slightly. Three litres of the culture fluid were centrifuged at 6000 g for 30 min at 4 °C to collect the cells. This cell pellet was suspended in minimum amount of 13 mM KH_2PO_4/115 mM NaCl/1 mM $MgSO_4$ buffer, pH 7.0, centrifuged at 6000 g for 30 min at 4 °C to collect the cells, then resuspended in minimum amount of 10 mM PBS, pH 7.0. After addition of DNase II to a final concentration of 1 μg/ml, the suspension was sonicated, then incubated on ice for 1 h. This mixture was centrifuged at 20 000 g for 30 min at 4 °C to remove the cell debris. The supernatant was centrifuged at 60 000 g for 1 h at 4 °C.

The crude enzymes were purified as follows. They were dialysed against three changes of 10 mM PBS buffer, pH 7.0. A sample of each was then purified by cation-exchange chromatography through a 5 ml Hi-Trap SP column. β-Lactamase was eluted with 0–0.5 M NaCl and pH 7.0–8.0 linear gradients. Upon dialysis of the desired fractions against three changes of 100 mM PBS, pH 7.0, further purification was achieved by gel filtration through Superdex 75 equilibrated with 100 mM PBS, pH 7.0. All enzymes were purified to more than 95 % homogeneity as judged on SDS/PAGE.

Matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS analysis

The protein was dialysed against ten changes of water. The de-salted protein was freeze-dried. For tryptic digestion, the protein was dissolved in 50 mM ammonium bicarbonate buffer, pH 7.9, at a concentration of 1 mg/ml. Promega Sequencing Grade Modified Trypsin was dissolved in the same buffer and added at an enzyme/substrate ratio of 1:100 (mol/mol). The solution was incubated at 37 °C for 9 h. The digestion was stopped by cooling in liquid nitrogen and the mixture was freeze-dried. The dried sample was dissolved in aq. 0.1 % trifluoroacetic acid at a concentration of 10 pmol/μl.

Mass spectra were obtained by MALDI-TOF MS using a Voyager Elite mass spectrometer equipped with a delayed-extraction system (PerSeptive Biosystems, Framingham, MA, U.S.A.). The samples were ionized by a nitrogen laser at 337 nm. The samples were analysed in the reflector and linear modes with α-cyano-4-hydroxycinnamic acid as a matrix. Data were analysed using GRAMS/386 software (Galactic Inc., Salem, NH, U.S.A.).

NMR titration sample preparation

A protein concentration of 1 mM was used for the wild-type enzyme and 0.5 mM was used for the K67C and Y150C mutants. Samples were dissolved in 0.4 ml of ^2H_2O containing 5 mM PBS, and the pH was adjusted by the addition of small amounts

(1–10 μl) of 1 M or 100 mM NaOH or HCl solutions. Samples of small model compound **1** (Figure 2 below) were also prepared by a similar procedure.

NMR spectroscopy

The pH was measured before and after each spectrum was recorded. The latter reading was used in data analysis. The pH meter was calibrated prior to use with pH 4.01, 6.86, and 9.18 standard solutions. The estimated uncertainty in pH measurement is 0.02 pH unit. Since the water content varied during titration, the meter readings were not corrected for isotope effect.

The ^{13}C-NMR spectra of the enzymes were measured at 180.62 MHz using a Bruker DMX-750 spectrometer. The one-dimensional spectra were obtained by 1024–4096 scans at 298.0 K under the WALTZ decoupling condition of proton. The flip angle of pulse was set to 30°, and 1.5 s of the repetition delay was used. The data size was 64 K words and the spectral width was 315.5 p.p.m. The obtained free induction decays ('FIDS') were processed by XWINNMR software version 2.5 (Bruker Analytik GmbH, Karlsruhe, Germany) using 10 Hz of exponential line-broadening factor. A small amount of dioxan was added to the sample, and its peak was used as an internal standard of 67.8 p.p.m.

The ^{13}C-NMR spectra of aqueous solutions of the small-molecule model compound **1** at various pH (1.75–11.24) were measured at 125.76 MHz using a Bruker DMX-500 spectrometer. A peak of dioxan was used as an internal standard of 67.80 p.p.m.

Solvent-accessibility calculation

Solvent-accessible surface areas were calculated with the program DMS, which is based on the Richards algorithm [22] and implemented in the program suite Midas plus [23]. The radius of the water probe was 1.4 Å (1 Å = 0.1 nm), and the density of points on the surface was 5 points/Å². Three X-ray structures (2BLT, 2BLS, 1GCE) [2–4] were obtained from the Protein Data Bank. All water molecules were removed from the structures before the calculations. When more than one molecule was present in the asymmetric unit, we calculated each of their surface areas.

RESULTS

The tyrosine residues of wild-type, K67C and Y150C mutants of class C β-lactamase from *C. freundii* were labelled specifically with L-[*phenol*-4-^{13}C]tyrosine. The ^{13}C label was incorporated at this position because this aromatic carbon atom is expected to be the most sensitive to changes in the protonation state of the adjacent phenolic oxygen atom.

MS confirmed the selective labelling of the tyrosine residues. The molecular mass of the labelled wild-type enzyme was 15 mass units heavier compared with the unlabelled protein (results not shown). In order to estimate the ^{13}C content of the tyrosine residue, labelled and non-labelled β-lactamase protein samples were digested with trypsin and subjected to MALDI-TOF MS. One of the digested fragments consisted of residues 149–176, containing two tyrosine residues (Tyr^{150} and Tyr^{170}). Figure 1 shows that the ion peak (m/z 2965.46) for the labelled β-lactamase (149–176) fragment, including two [*phenol*-4-^{13}C]tyrosine residues, was exactly 2 Da higher compared with the ion peak (m/z 2963.38) for the non-labelled β-lactamase (149–176), indicating the quantitative ^{13}C enrichment for the labelled fragment.

150   170
L Y ANSSIGLFGA LAVKPSGMS Y EEAMTR
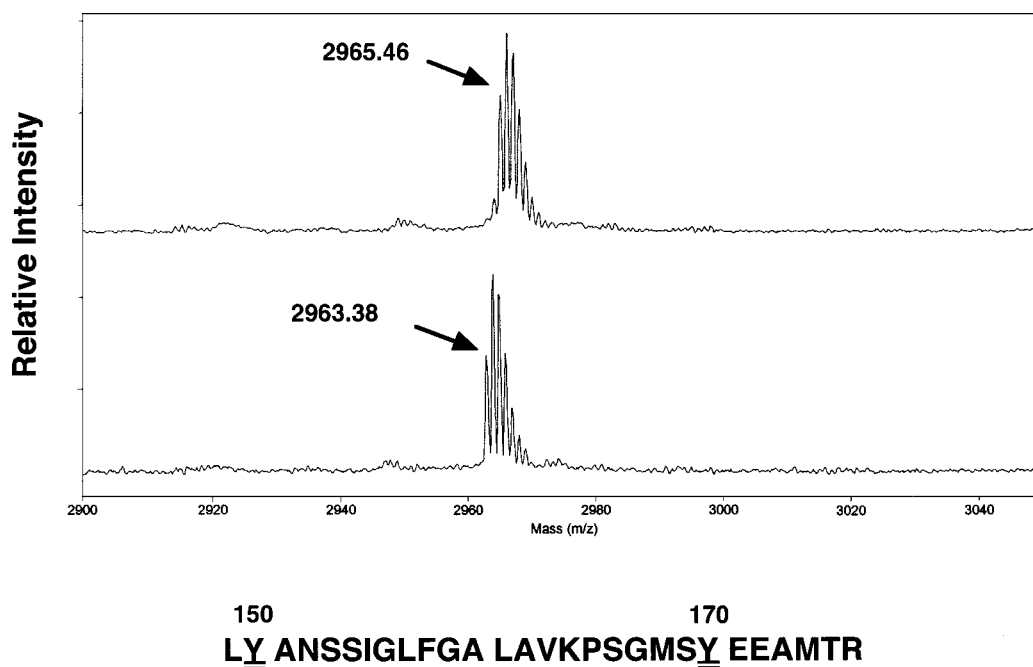
Figure 1   Observed pattern of the MALDI-TOF mass spectra for the β-lactamase 149–176 fragment of the [*phenol*-4-^{13}C]tyrosine-selectively-labelled β-lactamase (top) and non-labelled β-lactamase (bottom) protein

The wild-type and mutant β-lactamases showed indistingui-shable spectra at a higher field [chemical shift (δ) 0.6 $\approx$ −0.6] over the pH range 7–11 from one-dimensional ^{1}H-NMR (results not shown). Thus ^{13}C-NMR spectra of the labelled lactamases were obtained from pH 6 until complete denaturation at pH values above 12. Meanwhile, spectra of the labelled L-tyrosine amino acid alone were obtained over the pH range 7–13. Titration of the free amino acid showed a normal titration curve (results not shown). Its pK_a value and chemical shifts of the protonated and deprotonated tyrosine residue were obtained from non-linear fits of the titration data to eqn (1), which is derived from the Henderson-Hasselbalch equation [20,24]:

$$\delta_{obs} = (\delta_{HA} + \delta_A - 10^{(pH-pK_a)})/(1 + 10^{(pH-pK_a)}) \qquad (1)$$

where δ_{obs} is the observed experimental chemical shift, δ_{HA} is the chemical shift of the protonated group, and δ_A is the chemical shift of the deprotonated group. The calculated pK_a for the labelled tyrosine residue was 10.3 $\pm$ 0.1. The pK_a value seems reasonable for a solvent exposed free tyrosine. The chemical shifts for the protonated and deprotonated forms were calculated to be 156.36 and 165.69 p.p.m. respectively.

The model molecule **1** (Figure 2) showed chemical shifts at 152.64 and 161.63 p.p.m. for the protonated (pH 6.2) and deprotonated forms (pH 8.3) respectively. The higher-field shifts (about 4 p.p.m.) of the model molecule **1**, compared with free tyrosine, are reasonable for the 2,6-bisaminoalkyl-substituted phenol. Despite a considerable difference of the pK_a values between free tyrosine and the model compound **1** (Figure 2), it is apparent that the two positive ammonium groups do not appreciably affect the chemical shift of the deprotonated form.

Since the wild-type and K67C mutant enzymes contain 15 tyrosine residues, and Y150C has 14 tyrosine residues, the extra peak in the wild-type and K67C spectra should correspond to the signal from Tyr[150]. Also, comparing the spectra for wild-type and
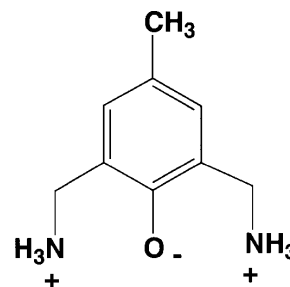
Figure 2   Small model compound 1

K67C at lower pH values, all peaks are at similar positions, except that the supposed Tyr[150] peak in the K67C mutant enzyme is at slightly higher field (Figure 3). The Tyr[150] peaks in wild-type and K67C lactamases identified in this manner were the second lowest-field tyrosine peaks at pH 7, and the chemical shifts (157.97 and 157.68 p.p.m.) indicate that the Tyr[150] residues are protonated at physiological pH. These chemical shifts are similar to that (158.36 p.p.m.) of the protonated form of free tyrosine. The second lowest-field peaks imply that the carbon adjacent to the hydroxy group is more deshielded compared with other similar carbon atoms. This in turn suggests that the adjacent phenolic oxygen atom is slightly more negatively charged and is likely to have a lower pK_a compared with other tyrosine residues, except for the unidentified tyrosine residue, which shows the lowest-field peak at 158.5 p.p.m. However, unexpectedly, the Tyr[150] peak in wild-type lactamase hardly shifted to a lower field even beyond pH 11, meaning that its pK_a values would be well over 11 (Figures 4 and 5). Beyond pH 12,
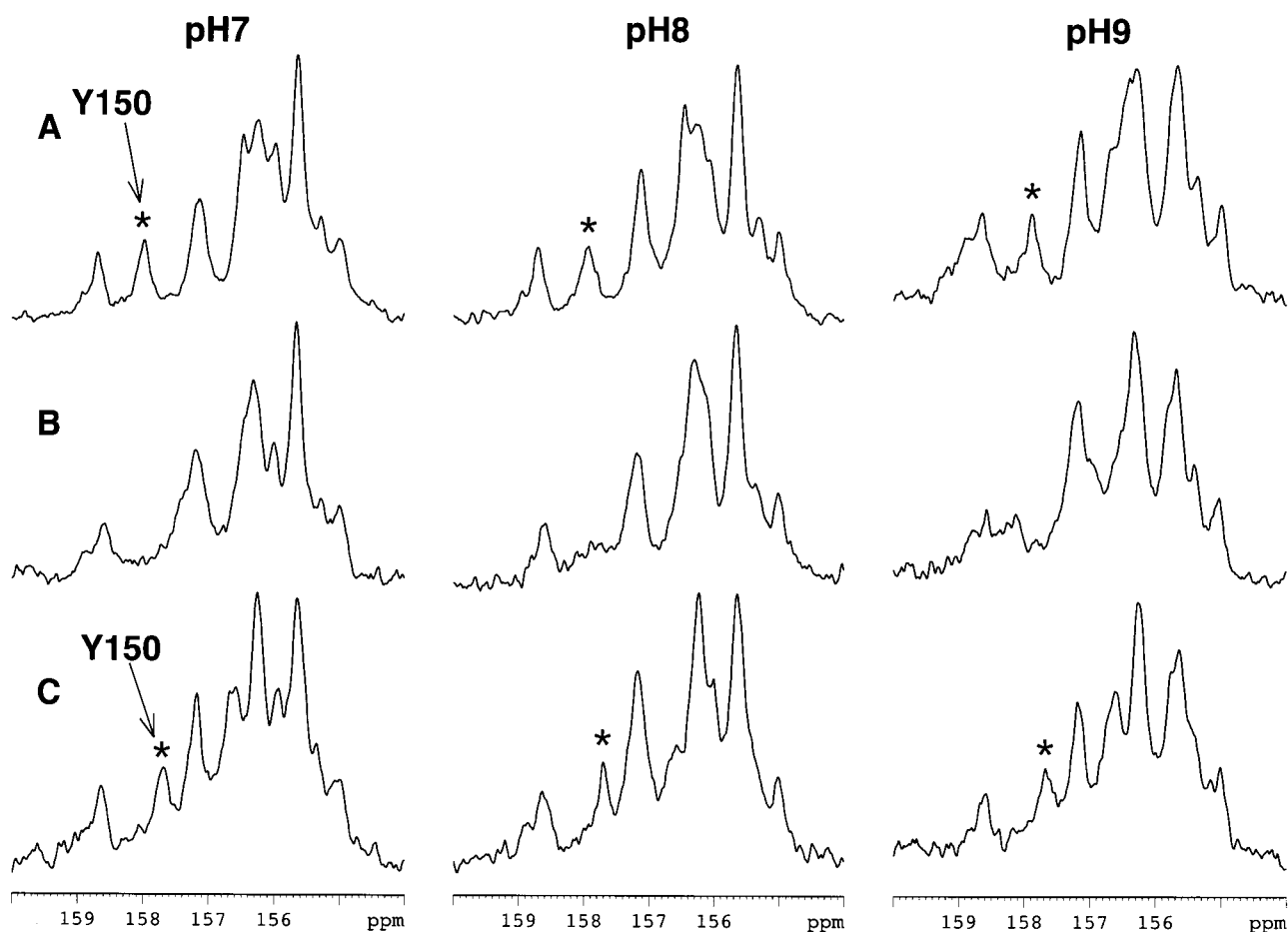
Figure 3   Comparison of wild-type (A), Y150C (B) and K67C (C) β-lactamase ^{13}C-NMR spectra at pH 7, 8 and 9

The peaks for Tyr^{150} are indicated by asterisks.

the protein showed only single peak at 166.77 p.p.m. for all the labelled carbon atoms of tyrosine residues, indicating that the enzyme is completely denatured. An estimate of the pK_a value for wild-type lactamase upon extrapolating the titration curves to eqn (1) was 13.1, assuming a chemical shift at 166 p.p.m. for the dissociated Tyr^{150} residue. Since the enzyme is completely denatured at pH 12.1, the pK_a of 13.1 should be only a formal value, though it would be more than 11. The Tyr^{150} peak in K67C mutant also showed no lower-field shift until pH 11.

DISCUSSION

Our previous results obtained from small-molecule model compounds indicated that the phenolic hydroxy group mimicking Tyr^{150} at physiological pH (7.0) is deprotonated [16]. Model compound **1** shows a chemical shift (161.62 p.p.m.) for the C-1 position of a deprotonated form, whereas a protonated form of compound **1** shows a higher chemical shift (152.64 p.p.m.) at a lower pH (6.2). In contrast, the pH titration results indicate that Tyr^{150} phenolic hydroxy-bearing carbon atom in the actual enzyme does not show a lower shift at pH 7.0–10.0. Beyond pH 12 the enzyme was denatured and Tyr^{150} shifted to a lower field (165.77 p.p.m.). This lower field shift was similar to those of the small-molecule model and free tyrosine. This result suggests

that the environment surrounding the Tyr^{150} side chain favours a protonated form and is consistent with the crystallographically observed hydrogen bond between the carboxylate of the cephalosporin derivative and Tyr^{150} of a class C β-lactamase mutant [18]. One reason for this behaviour of Tyr^{150} in wild-type lactamase may be its low solvent accessibility. The two distinct conformations of the Tyr^{150} residue are found in the crystal structure of AmpC β-lactamase from *Enterobacter cloacae* P99 (2BLT) [2], in which the phenolic oxygen atom of Tyr^{150} shows a hydrogen bond with Lys^{67} in one structure (B-chain of 2BLT) and with Lys^{315} in another (A-chain). Solvent-accessibility calculations [22] at the phenolic hydroxy group calculated for the crystal structure indicate that the phenolic oxygen atom of Tyr^{150} in the A-chain has low solvent accessibility (1.30 Å^2), whereas phenolic oxygen in the B-chain is more solvent-accessible (2.26 Å^2). The phenolic hydroxy group in another crystal structure of the class C β-lactamase (2BLS) [3] from *Escherichia coli* forms a hydrogen bond with Lys^{315} and has low solvent accessibility (1.79 Å^2 and 1.68 Å^2), whereas the phenolic hydroxy group in a natural β-lactamase mutant (1GCE) [4] forming a hydrogen bond to Lys^{67} has higher solvent accessibility (11.21 Å^2). On the other hand, Lys^{67} and Lys^{315} are not solvent-exposed in the β-lactamases. No matter how electronically susceptible a proton is to deprotonation, if its conformation does
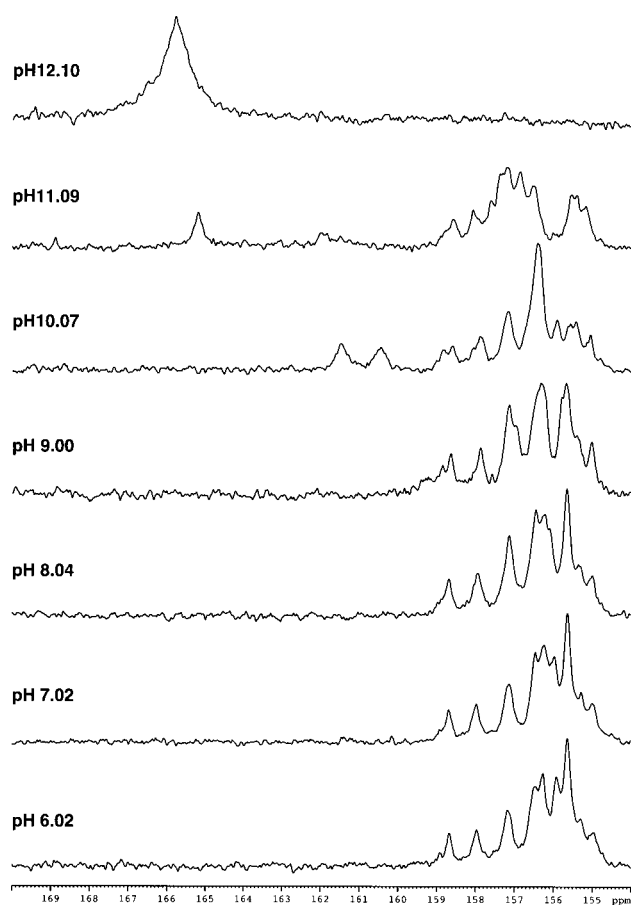
Figure 4   Wild-type β-lactamase ^{13}C-NMR spectra at various pH values
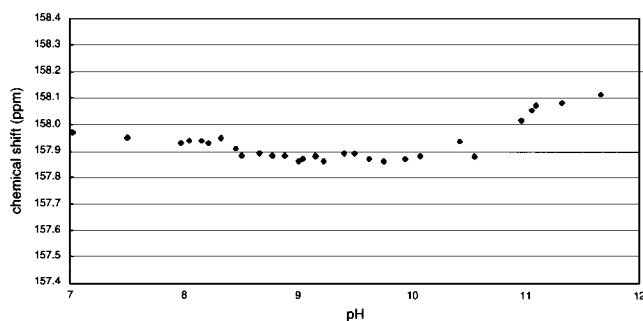

Figure 5   Titration plots for wild-type β-lactamase in 5 mM PBS at pH 7–12

Plots at higher pH are not shown because of denaturation of the enzyme.

Tyr[150]. This abstraction of the phenolic proton at pH 8.3 is consistent with the pK_a calculated by the Poisson–Boltzmann method for Tyr[150] [19]. The cationic Lys[67] or Lys[315], which is hydrogen-bonded to the phenolic oxygen atom, simultaneously may neutralize the phenolic anion and the cationic amine itself also becomes neutral. The crystal structures of class C β-lactamases suggest that the phenolic proton of Tyr[150] is buried when Tyr[150] is hydrogen-bonded to Lys[315], whereas it becomes exposed to the solvent when a hydrogen bond is formed between Lys[67] and Tyr[150]. It is plausible that Lys[67] is protonated at physiological condition, as observed in class A β-lactamases [25], because the N$^\zeta$ atom of Lys[67] locates within hydrogen-bond distances to Ser[64], Tyr[150] and Asn[152] in the crystal structure (A-chain of 2BLT). The proton transferred from the protonated Lys[67] to the phenolate group of Tyr[150] may then be completely buried, since it may be pointing to the neutralized Lys[67] and away from the surface. If so, the phenolic hydroxy group may not be deprotonated at pH 8.3–11. At pH values beyond 11, a global conformational change of the enzyme may begin, and the enzyme is completely denatured beyond pH 12. Thus the fairly high value of the estimated pK_a (more than 11) for Tyr[150], compared with the pK_a value (10.3) of free tyrosine, suggests that the phenolic proton is well buried and becomes exposed upon global conformational change of the protein. The low solvent accessibilities of Tyr[150] and the neighbouring two lysine residues suggest that protons of these residues are hardly exchanged with those of the solvent at physiological pH. It was reported that no, or only small, solvent ^{2}H kinetic isotope effects had been observed in acyl-enzyme formation in class C β-lactamase [26]. This observation seems to be consistent with the present result on pK_a of Tyr[150] and the low solvent accessibilities of Tyr[150] and the neighbouring residues.

The substitution of a neutral residue for Lys[67] may yield a mutant in which the phenolic oxygen atom of Tyr[150] would form a hydrogen bond only with Lys[315] and the phenolic proton of Tyr[150] would be buried at pH 7–11, as observed in the native enzyme at pH 8.3–10.6. The mutant surely maintained a protonated Tyr[150] residue up to pH 10.4, showing a similar [*phenol*-4-^{13}C]Tyr[150] peak at pH 7.0–10.4 with a slightly higher field shift overall (about 0.2 p.p.m. from the peak of the native enzyme at pH 9.0). This result indicates that the neighbouring positive charge has only a small effect on the chemical shift of [*phenol*-4-^{13}C]Tyr[150].

The present results on the protonated states of Tyr[150] are quite different from the result observed in the small model compounds [16]. On the other hand, the results are consistent with the hydrogen bond observed in the crystal structure of the cephalosporin-bound mutant enzyme complex [18]. The phenolate anion of the small model compounds should be exposed to solvent in aqueous solution and would form an ion-pair with Na$^+$ ion above pH 7. This ion-pair would be separated from the positively charged amines by solvation. Consequently, the phenolate and the positively charged amines would co-exist in the aqueous solution. On the other hand, the phenyl group of Tyr[150] at a low solvent-accessible surface of the enzyme is proximal to the neighbouring protonated lysine residues. However, these two protonated residues may not stabilize an ionized form of Tyr[150], but will transfer a proton to the phenolate anion, since the basicity of a naked (unsolvated) phenolate anion would be too strong for maintaining an ionic pair with the protonated cation. Thus the native and mutant enzymes will favour a protonated form of Tyr[150] at physiological pH.

In the hydrolysis of penicillins catalysed by class C β-lactamases it has been observed that the ionization in the acidic limb of the pH profile is about 6 [15]. There are two candidate groups
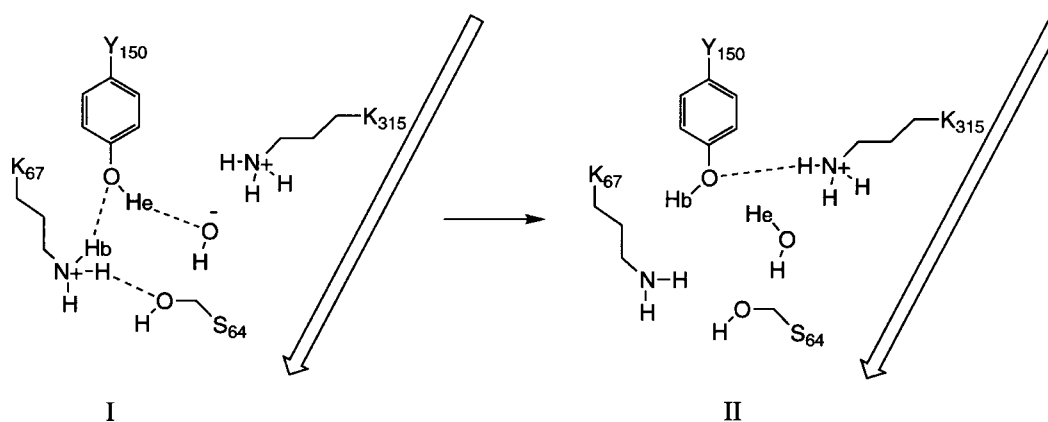
not allow access to its surrounding solvent, it would be difficult to remove that proton, and its pK_a would rise. Interestingly, the titration plots indicate a slight high-field shift (about 0.1 p.p.m.) in the region between pH 8.3 and 10.6 in the wild-type enzyme, as shown in Figure 5. This may be due to changes of the electronic state in the vicinity of the Tyr[150] side chain, and this change may involve proton shifts. Thus it is most likely that, at pH 8.3, a base ($^-$OH from the solvent) abstracts a proton from

Scheme 1   Plausible proton transfer around pH 8.3

Open arrow indicates the conserved β-strand at Lys^{315}–Thr^{318}. Broken lines indicate putative hydrogen bonds. Hb and He indicate the buried and exposed protons respectively. When the base approaches Tyr^{150}, Tyr^{150} forms a hydrogen bond with Lys^{67} (**I**). Concomitantly with a proton abstraction from Tyr^{150}, a proton from Lys^{67} would replace it (**II**).

responsible for the acidic limb. One is the phenol group of Tyr^{150} and the other is the carboxylate group common in the substrates of the enzyme. The present results indicate that Tyr^{150} is not the responsible residue, since [*phenol*-4-^{13}C]Tyr^{150} at pH 6.02 showed the chemical shift at 157.97 p.p.m. (Figure 4). Thus the carboxylate group could be a candidate responsible for the acidic limb.

The results are summarized in Scheme 1. When the base approaches Tyr^{150}, Tyr^{150} forms a hydrogen bond with Lys^{67} (I). Concomitantly with a proton abstraction from Tyr^{150}, a proton from Lys^{67} would replace it, and the resulting Tyr^{150} phenolic proton would remain near its original position (II). Since Lys^{67} is not exposed to the aqueous phase, this Tyr^{150} phenolic proton is expected to be completely buried from the solvent. Consequently, Tyr^{150} would appear protonated until the enzyme is denatured. As the actual pK_a of the Tyr^{150} residue can be estimated as 8.3, the positively charged Lys^{67} and Lys^{315} residues contribute to a more than two order of reduction of pK_a of the tyrosine residue. The present results suggest that Tyr^{150} does not directly participate in the activation of Ser^{64} as a general base and may afford another view for the activation of Ser^{64} [27].

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