Enzyme-Induced Strain/Distortion in the Ground-State ES Complex in β-Lactamase Catalysis Revealed by FTIR†

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ABSTRACT: Class A β-lactamases hydrolyze penicillins and other β-lactams via an acyl−enzyme catalytic mechanism. Ser70 is the active site nucleophile. By constructing the S70A mutant, which is unable to form the acyl−enzyme intermediate, it was possible to make stable ES complexes with various substrates. The stability of such Michaelis complexes permitted acquisition of their infrared spectra. Comparison of the β-lactam carbonyl stretch frequency (ν_{CO}) in the free and enzyme-bound substrate revealed an average decrease of 13 cm^{-1}, indicating substantial strain/distortion of the lactam carbonyl when bound in the ES complex. Interestingly, regardless of the frequency of the C═O stretch in the free substrate, when complexed to *Bacillus licheniformis* β-lactamase, the frequency was always 1755 ± 2 cm^{-1}. This suggests the active site environment induces a similar conformation of the β-lactam in all substrates when bound to the enzyme. Using deuterium substitution, it was shown that the "oxyanion hole", which involves hydrogen bonding to two backbone amides, is the major source of the enzyme-induced strain/distortion. The very weak catalytic activity of the S70A β-lactamase suggests enzyme-facilitated hydrolysis due to substrate distortion on binding to the enzyme. Thus the binding of the substrate in the active site induces substantial strain and distortion that contribute significantly to the overall rate enhancement in β-lactamase catalysis.

β-Lactamases are the major source of resistance to β-lactam antibiotics and have been the targets of considerable mechanistic scrutiny. Despite numerous investigations, many details of the catalytic mechanism are still uncertain. The residues believed to be most important in catalysis by the class A family of β-lactamases include the strictly conserved active site residues, Ser70 and Lys73, as well as Glu166, Asn170, Ser130, and Lys234 (*1*). With the exception of Ser70, which acts as a nucleophile to form a covalent acyl−enzyme intermediate (Scheme 1), the mechanistic role of the other residues remains controversial. Kinetic studies indicate that catalysis is dependent on at least two ionizable groups with p*K*'s in the vicinity of 5 and 8.5. Glu166 is a likely candidate for the observed ionizable group with a pK_1 in the vicinity of 5. The p*K* and the heat of ionization of the group responsible for the alkaline limb of the pH−rate profile have been shown to be consistent with a lysine residue (*2*), and Lys73 is a logical choice for the group responsible for pK_2. In fact, using β-lactamase mutants, in which Lys73 and Lys234 were mutated to alanine, we have recently shown that pK_2 reflects the ionization of both Lys73 and Lys234 (Lietz E., et al., submitted for publication). Such an assignment would require the catalytically active form to be the protonated state. Substantial evidence supports an acyl−enzyme kinetic mechanism (Scheme 1) in which the acyl−enzyme bond is formed by nucleophilic attack of the side chain of Ser70 on the β-lactam carbonyl (*1*).

Scheme 1

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P$$

Ground-state distortion toward the transition state is believed to be a significant component of an enzyme's catalytic machinery; however, examples that directly demonstrate such distortion are few. In the present investigation, we have used a combination of site-directed mutagenesis and FTIR spectroscopy to probe the structure of the noncovalent Michaelis complex. This was achieved by making the S70A mutant of β-lactamase, in which the active site serine is replaced by alanine, preventing formation of the acyl−enzyme and thus stopping the reaction at the ES complex, and by examination of the CO stretch frequency of the β-lactam carbonyl. By using hydrated thin-film attenuated total reflectance (ATR)[1] FTIR it was possible to use much lower enzyme concentrations in the reaction solution than those required in typical FTIR transmission mode measurements (e.g., ≤1 mg/mL as compared to >10 mg/mL). It has been previously shown that the hydrated thin-film ATR technique effectively does not affect the structure of native proteins (*3*−*6*). This is because it is only the first layer of protein in contact with the IRE that may be structurally perturbed, and this contributes a negligible amount to the total signal. Fortuitously, neither the enzyme nor the product penicilloic acids absorb in the 1700−1800 cm^{-1} region, whereas substrates have a prominent absorbance due to the β-lactam carbonyl in the vicinity of 1760−1780 cm^{-1}. Thus it should be possible to trap noncovalent ES complexes using

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[1] Abbreviations: FAP, *N*-(2-furylacryloyl)penicillin; WT, wild-type; IRE, internal reflectance element; ATR, attenuated total reflectance.

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the S70A enzyme and, from analysis of the carbonyl β-lactam band position, determine whether the enzyme induces strain or distortion of the β-lactam carbonyl bond in the complex.

MATERIALS AND METHODS

β-Lactamase from *Bacillus licheniformis* was produced and purified by overexpression from *Escherichia coli* as described elsewhere (Lietz, E. J., and Fink, A. L., manuscript submitted). Enzyme preparations used were homogeneous by IEF−PAGE with Coomassie Blue staining. Benzylpenicillin, cefoxitin, cefotaxime, phenoxymethylpenicillin, lysozyme, bovine pancreatic γ-chymotrypsin, and IPTG were purchased from Sigma. *N*-(2-furylacryloyl)penicillin (FAP) was from Calbiochem.

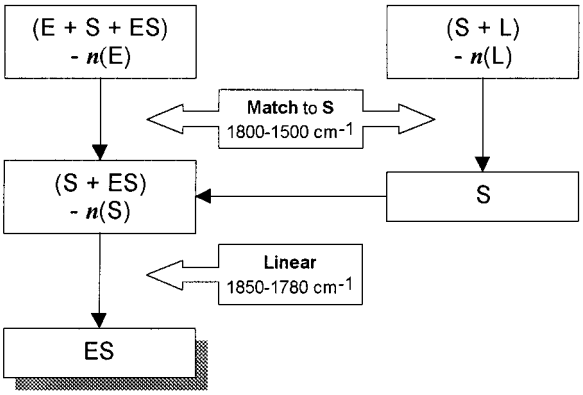
Enzyme Kinetics. β-Lactamase activity toward FAP was determined spectrophotometrically using a Hewlett-Packard 8452A spectrophotometer. Path lengths employed were 1.0 or 0.1 cm. The hydrolysis of the amide bond in the lactam ring was followed at 340 nm, where $\epsilon = 3460$ M^{-1} cm^{-1} and $\Delta\epsilon = 1905$ M^{-1} cm^{-1}. Data analyses were performed using SigmaPlot (SPSS, Chicago, IL). For determination of K_i and K_m, initial velocities were used. The pH dependence of k_{cat}/K_m was determined under first-order conditions (where $K_m \gg$ [S]). All assays were run at 30 °C, and the S70A concentration was usually 15 μM. Reactions were buffered by sodium acetate (pH 4.0−5.5), sodium phosphate (pH 6.0−8.0), sodium pyrophosphate (pH 8.0−9.5), and CAPS (pH 10.0−10.5). Buffer concentrations for all kinetic assays were 125 mM, with 0.5 M KCl. The rate of spontaneous hydrolysis of the substrates was subtracted from the enzyme-catalyzed rates. Ionization constants were determined by fitting the (k_{cat}, K_m, and k_{cat}/K_m) pH profiles to the expression in eq 1.

$$k_{obs} = k_{lim}/(1 + [H^+]/K_1 + K_2/[H^+] + K_2/K_1) \quad (1)$$

Sample Preparation for FTIR. Freshly prepared solutions of β-lactam substrates (penicillins and cephalosporins) were made in either DDI H_2O or D_2O, depending on the experiment performed, containing 10 mM potassium phosphate buffer (pH 7.0). To prevent significant substrate hydrolysis, the S70A β-lactamase mutant was allowed to react with a substrate for only 2−3 min prior to analysis. Variable incubation times were used in turnover experiments with wild-type enzyme.

Typically, the concentration for each constituent was as follows: 450 μM substrate and 45 μM S70A β-lactamase (or 45 μM chicken egg white lysozyme or bovine pancreatic γ-chymotrypsin; see below). In some cases, 1:1, 2:1, or 4:1 substrate/enzyme ratios were used, with 45 or 90 μM enzyme. The buffer was 10 mM potassium phosphate, pH 7.0. All reaction mixtures were incubated at 22 °C. In the ATR experiments, samples (50 μL) were placed on a clean internal reflectance element (IRE), and the solvent was evaporated under a gentle stream of nitrogen gas. The substrate solution was either mixed with enzyme stock solution in a small vial and aliquots were removed for ATR-FTIR analysis at the desired time intervals, or more typically the solutions were mixed directly on the IRE. During the solvent evaporation, a pipet tip was used to spread the sample uniformly over the surface of the IRE. Since the mixing time of β-lactamase with substrate is crucial, steps were taken to minimize their time of exposure. These steps include the use of 50 μL samples as well as mixing the β-lactamase enzyme with substrate on the crystal. Once the bulk solvent had been removed, the IRE was placed in the FTIR sample compartment, and a spectrum was collected. The nitrogen used to dry deuterated samples was bubbled through deuterium oxide to prevent ^{1}H exposure to the sample while drying.

Scheme 2: Subtraction Protocol Used to Extract the ES Spectrum[a]



[a] E is the β-lactamase, S is substrate, L is lysozyme (see text), (E + S + ES) is the reaction mixture of enzyme and substrate, and ES represents the substrate bound in a Michaelis complex with the β-lactamase. At each step in the process, the subtraction scaling factor (n) was iteratively optimized by SAFAIR using the criteria indicated to evaluate the subtraction result.

Deuterated Sample Preparation. β-Lactamase (S70A) and lysozyme were dialyzed against DDI water and lyophilized and then were dissolved in an appropriate volume of D_2O, centrifuged, and the supernatant was lyophilized. Lyophilization and dissolution in D_2O were performed twice more to ensure complete solvent exchange.

FTIR Spectroscopy. Spectra were obtained using a Nicolet 800SX FTIR spectrometer equipped with an MCT detector. A total of 1024 interferograms were collected at either 4- or 2-cm^{-1} resolution. In ATR analysis, samples were examined on a trapezoidal ZnSe IRE crystal (73 × 10 × 6 mm, 45°) placed in a horizontal out-of-compartment ATR apparatus (SPECAC) (*3*). For some experiments, a germanium IRE was used.

FTIR Data Processing and Analysis. All the data were analyzed with GRAMS/32 from Galactic Industries. The interferograms were converted to spectra with the Mertz method using medium Norton−Beer apodization. Contributions from water vapor, buffer, and solvent water were removed by subtraction. Two to four independent spectra of each sample type were scanned and examined individually to ensure consistent results.

Subtractions were used to identify the spectral changes induced by the enzyme on the β-lactam carbonyl. The overall subtraction protocol is outlined in Scheme 2. In essence, this describes the subtraction of both the enzyme and the unbound-substrate signals from the spectrum of the mixture.

In practice, obtaining reproducible results from these subtractions was not straightforward. Although signals are strong in ATR-FTIR, their intensities vary from sample to sample due to slightly different distributions of a sample on the surface of the IRE. One of the spectra must always be rescaled to match the other. To overcome this variability and still obtain the objectivity of an automatic processing

algorithm, the SAFAIR subtraction software package was employed. SAFAIR iteratively varies the scaling factor for a subtraction such that a user-selected "goodness" function is optimized (minimized). SAFAIR is well-suited to the extraction of small signals from intense spectra because it optimizes the subtraction-scaling factor based on the resulting difference spectrum rather than on the original spectra. The RMS deviation of replicate subtractions for a given substrate was on the order of ±2%.

The SAFAIR criteria used for this analysis were "linear" and "match". The match function optimizes the subtraction so that the shape of the difference (result) spectrum is as close as possible to a reference spectrum. Here, the reference spectrum was that of the substrate alone. The subtractions therefore removed all protein signal and left only the substrate signal. Note that there was a small contribution from the ES complex in the reaction mixture; however, this band was no more than 5−10% of the intensity in a small window of the data region that was used for the optimization. This signal can therefore be considered negligible in the result spectrum (S + ES). The linear function is optimal when the subtraction result is a straight line. Linearizing the 1850−1780 cm^{-1} data region caused a scaling factor to be selected that brought the slope of a region where there is only substrate signal (1800−1780 cm^{-1}) to the same value as a baseline region (1850−1800 cm^{-1}). This effectively removed the substrate signal without oversubtracting.

Because of the effects of the refractive index of the protein on the substrate spectrum, which causes small shifts (<3 cm^{-1}) in the substrate band position (see Results), the substrate spectrum used was a difference spectrum of a substrate/lysozyme mixture minus lysozyme, not a spectrum of the substrate alone. This subtraction procedure was also carried out with γ-chymotrypsin instead of lysozyme to demonstrate that there was no unique effect of lysozyme on the substrate. Product spectra were obtained in a fashion similar to those for substrates, from hydrolysis of the substrate using 10−100 nM wild-type β-lactamase.

The turnover reaction between wild-type β-lactamase and cefoxitin was monitored by incubating enzyme and substrate at 30 °C, pH 7.0. At various time points after initiation of the catalytic reaction, aliquots were removed and dried on the IRE to form a hydrated thin-film prior to spectral data acquisition. The time from removal of the aliquot to the dried state was 2 min.

RESULTS

The absence of the critical nucleophilic hydroxyl of Ser70 in the S70A mutant means that the normal catalytic mechanism involving a covalent acyl−enzyme (Scheme 1), as observed with the wild-type enzyme, cannot occur with this mutant. Since the replacement of the serine side chain by the methyl group of the alanine would not be expected to affect the binding of substrate, one would expect a noncovalent Michaelis complex to be formed between the mutant enzyme and the β-lactam substrates. Since other enzymes with comparable mutations have been found to have low levels of enzyme-facilitated hydrolysis of substrates, it was important to first determine the catalytic properties of the S70A mutant as well as to confirm that the mutation did not adversely affect the structural properties of the enzyme.

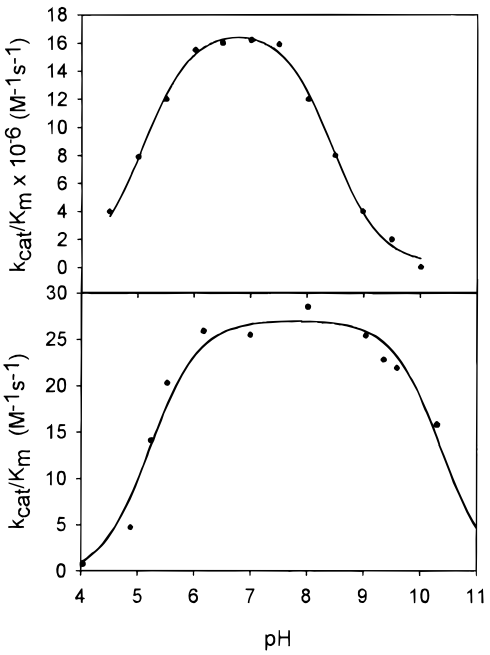


FIGURE 1: pH dependence of S70A β-lactamase catalysis. Top panel shows the data for wild-type *B. licheniformis* β-lactamase, the lower panel shows the data for the corresponding S70A mutant. The substrate was *N*-(2-furylacryloyl)penicillin, and the temperature was 30 °C. Values of k_{cat}/K_m were determined from complete progress curves under pseudo-first-order conditions. Note the million-fold difference in ordinate scales.

Properties of S70A β-Lactamase. The possible effect of the conversion of Ser70 to Ala on the structure of β-lactamase was investigated using far-UV circular dichroism, tryptophan fluorescence, and thermal stability. The CD and fluorescence spectra for the wild-type and S70A mutant were superimposable, indicating no significant perturbation of the structure of the enzyme. The thermal stability of the wild-type and S70A β-lactamases was compared using Trp fluorescence; at pH 7.0 the T_m was 65.0 ± 1.0 °C in both cases, indicating that the substitution had no effect on the stability of the protein.

The kinetic properties of the S70A mutant were examined in detail using the substrate FAP. The kinetics were complicated by product inhibition, which was shown to be competitive in nature, with an apparent K_i = 21 mM at pH 7.0, 30 °C. The K_m at pH 7.0, 30 °C, was estimated to be 14 mM from initial velocity measurements. The high values of K_m resulted in technical difficulties (e.g., limited substrate solubility as well as product inhibition) in attempts to measure k_{cat} and K_m separately as a function of pH. Consequently, the pH dependence of the reaction with FAP was ascertained using initial velocity measurements under pseudo-first-order conditions in which the effects of product inhibition were negligible due to the low substrate concentration (≤1 mM). A bell-shaped pH−activity profile was observed at 30 °C (Figure 1), with a broad pH optimum at around pH 7−9, and pK_1 = 5.2 ± 0.1, and pK_2 = 10.4 ± 0.1. The corresponding values for the wild-type enzyme are pK_1 = 5.1 ± 0.1, and pK_2 = 8.4 ± 0.1. Thus, in the mutant, K_m is increased ~10^3, and k_{cat}/K_m is decreased by ~10^6 relative to the wild-type β-lactamase, and there is a significant increase in pK_2 for the mutant. No significant buffer ion effects were noted when the acetate ion concentration

was varied over 2 orders of magnitude at pH 5.5, suggesting that buffer ion effects are not responsible for the observed pH dependence.

Since the S70A mutant lacks the critical active site nucleophile to form the covalent acyl−enzyme intermediate, the catalytic mechanism must be different in the S70A mutant as compared to the wild-type enzyme. The pH dependence of the mutant-catalyzed reaction, along with the significantly increased rate of substrate hydrolysis over spontaneous hydrolysis at pH <10, suggests that the enzyme nevertheless still catalyzes the reaction to a significant extent, presumably through enzyme-assisted hydrolysis. Most germane to the FTIR investigation is the fact that the turnover rate is very slow (<1 s^{-1}), allowing accumulation of the ES complex.

FTIR Spectra of β-Lactamase and β-Lactams. Most IR studies of enzyme−substrate complexes have used the transmission mode and D_2O as solvent. The large IR absorbance of liquid water necessitates very short sample path lengths and thus very high protein concentrations; previous investigations have usually used 50−300 mg/mL protein solutions (*7*), and even the latest high-sensitivity instruments require on the order of 10 mg/mL or more (*8*). The ATR method of FTIR has many advantages and, with hydrated thin-films, can be used to provide excellent spectra of proteins with as little as 10−50 μg of protein (*3*, *5*). Consequently, we chose to collect the FTIR spectra using this technique. The spectrum of β-lactamase obtained in this manner was very similar to that obtained by transmission mode (the small changes observed are due to the optical properties of the ATR system), and analysis of the secondary structure using the amide I region indicated the conformation was the same as that determined by X-ray crystallography (*9*), within experimental error, namely, 47% α-helix, 19% turn, and 29% β-sheet. The enzyme in the hydrated thin film was also shown to be catalytically active. For example, when a sample of β-lactamase (the K73A variant) was deposited as a thin film on a ZnSe IRE under the same conditions as used in the ATR-FTIR experiments and then redissolved, 92 ± 2% of the catalytic activity was recovered in the solution.

We examined the structures of the ES complexes with S70A for six representative penicillins and four typical cephalosporins. For the unbound substrates in phosphate buffer, pH 7.0, the β-lactam carbonyl stretch was observed in the 1762−1771 cm^{-1} range. We observed that in the presence of protein there were small shifts (0−3 cm^{-1}) to lower frequencies in these bands, presumably due to the refractive index of the protein. To compensate for these, we collected the spectra of the free substrates in the presence of lysozyme at the same concentration as that of β-lactamase in the experiments described below. The positions of the β-lactam carbonyl stretch frequency under these conditions are shown in Table 1. As a control to determine that the protein effects were not specific, some of the spectra were also collected in the presence of γ-chymotrypsin. The resulting substrate spectra were identical to those in the presence of lysozyme. The substrates were hydrolyzed by the addition of a trace of wild-type enzyme, and the spectra of the resulting products were collected. Figure 2 shows the 1350−1800 cm^{-1} region of the spectra of the enzyme and a typical substrate and product, illustrating that only the substrate has significant absorbance in the 1700−1800 cm^{-1} region of the IR.

Table 1: Absorbance Peaks for Substrates and S70A β-lactamase ES Complexes[a]

substrate	substrate absorbance (cm^{-1})	ES complex absorbance (cm^{-1})
benzylpenicillin	1766	1757
furylacryloylpenicillin	1765	1755
ampicillin	1767	1753
carbenicillin	1764	1756
methicillin	1767	1756
nafcillin	1768	1757
cefotaxime	1769	1753
cefoxitin	1767	1754
cephaloridine	1774	1757
cephalosporin C	1764	1753

[a] Conditions were at pH 7.0; the estimated error in the band position is ±2 cm^{-1}.

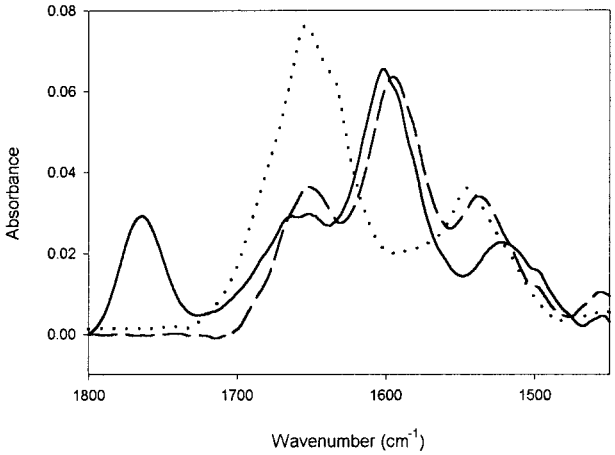


FIGURE 2: FTIR spectra of substrate, product, and β-lactamase showing that only the substrate has absorbance in the 1700−1800 cm^{-1} region. The substrate spectrum is shown as a solid line with the β-lactam carbonyl band at 1767 cm^{-1}, and the spectrum of the corresponding penicilloic acid product is shown as a dashed line; the product has no features in the 1700−1800 cm^{-1} region. The spectrum of the enzyme is shown as a dotted line. Spectra have been normalized to constant area (concentrations were 450 μM for substrate and product, and 45 μM for enzyme). As noted in the text, due to a refractive index effect from the presence of protein, the substrate and product spectra are actually difference spectra obtained in the presence of lysozyme. The substrate was carbenicillin, and data were collected in water (H_2O) at pH 7.0 (10 mM phosphate buffer).

FTIR Spectra of ES Complexes between S70A and β-Lactams. The procedure used to obtain the spectra of the ES complexes involved subtracting the spectral contributions of the free (unbound) substrate and the enzyme from the spectrum of the reaction mixture. In some cases, the spectrum of the product was also subtracted. However, the rate of hydrolysis was sufficiently slow under the experimental conditions that this step was found to be unnecessary, as there was negligible product present in the early stages of the reaction. Because the dissociation constant K_s was found to be quite high, it was necessary to use high substrate concentrations in the reaction mixture to attain close to saturation conditions. Because of limited enzyme solubility and to conserve enzyme, we chose to use excess substrate stoichiometry; typically, a 10-fold excess of substrate was used, with 45 μM final enzyme concentration. Under these

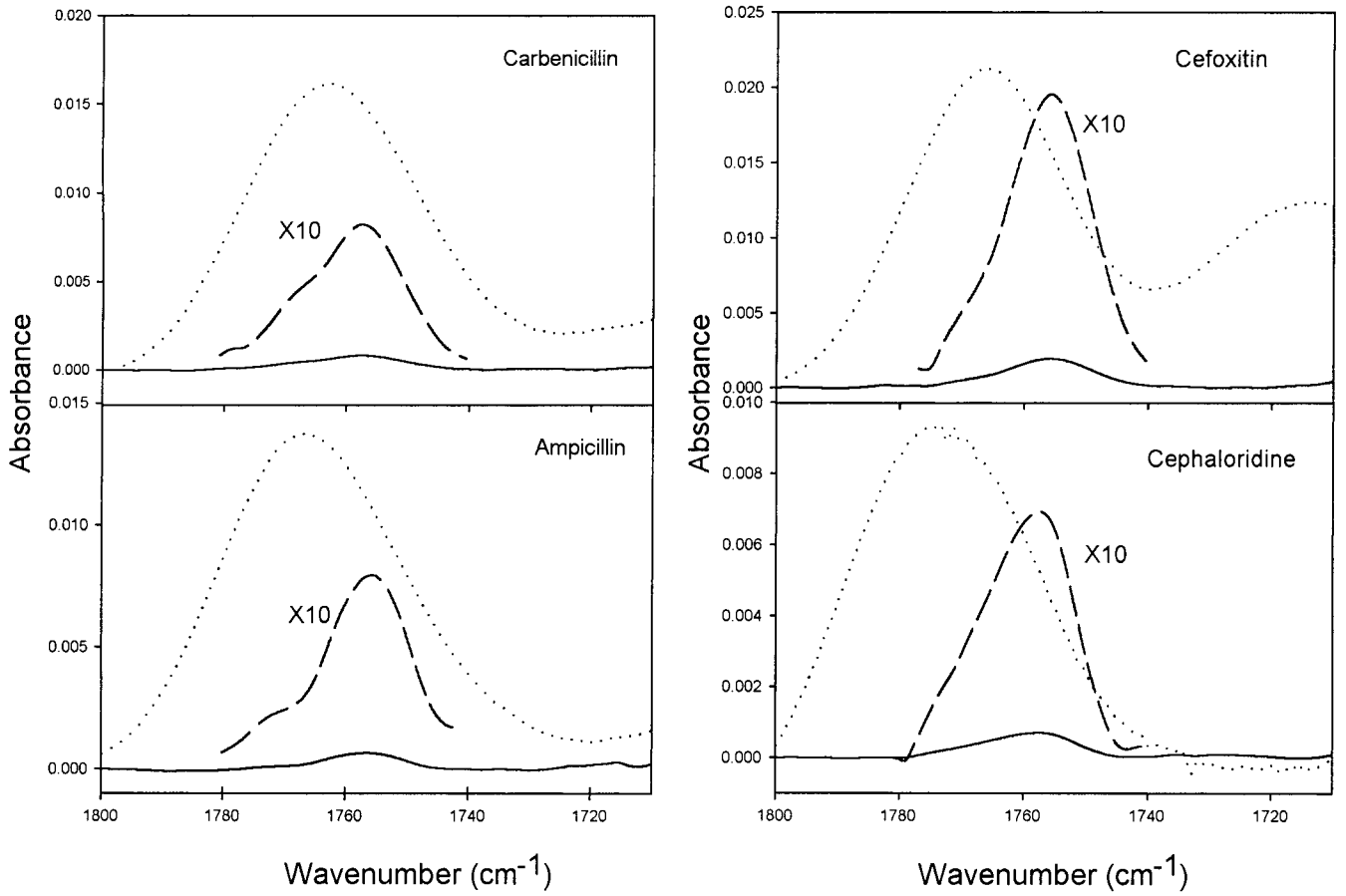


FIGURE 3: FTIR spectra of unbound substrates and noncovalent ES complexes. Spectra for the ES complexes were obtained by subtraction of the spectra of the enzyme and substrate from that of the mixture of S70A β-lactamase (45 μM) and substrate (450 μM), incubated for 2 min at 30 °C, pH 7.0. Penicillins (left panels: carbenicillin and ampicillin) and cephalosporins (right panels: cefoxitin and cephaloridine) all show bands at 1755 $\pm$ 2 cm^{-1} for the ES complex (solid lines), some 10−16 cm^{-1} lower frequency than those of the unbound substrates (dotted). Normalized ES spectra (i.e., converted to the same concentration scale as the substrate) are shown as dashed lines. Their areas are smaller, mostly due to less than 100% saturation of the enzyme.

conditions, the fraction of enzyme with bound substrate was usually in the 40−90% range, depending on the specific substrate.

Representative spectra of the substrates and ES complexes are shown in Figure 3. The difference spectra clearly revealed the presence of a new species, the noncovalent ES complex. The frequencies of the β-lactam carbonyl band in the ES complexes are given in Table 1. Interestingly, for all 10 substrates examined, the position for the ES complex was 1755 $\pm$ 2 cm^{-1}, representing an average of a 13 cm^{-1} decrease in frequency compared to the unbound substrates. Thus, within experimental error, all the noncovalent ES complexes have identical β-lactam carbonyl stretch frequencies, presumably indicating identical underlying structures in this critical region of the ES complex.

On the basis of examination of the enzyme's structure, it has been proposed that on binding the substrate, the β-lactam carbonyl will be located in an "oxyanion hole" formed by the backbone amides of residues Ser70 and Ala237 (*10*). This model predicts two hydrogen bonds between the carbonyl and the enzyme in the ES complex. If this is correct, then replacement of the amide hydrogens with deuterium should lead to stronger hydrogen bonds to the carbonyl and a shift to lower frequency in the FTIR spectrum. This is because the greater mass of deuterium relative to hydrogen, resulting in a lower zero point energy for deuterium bonds, leads to a decrease in the frequency of the C=O stretch vibration and a stronger hydrogen bond (*11*). Thus, the preceding experiment was repeated using deuterated S70A β-lactamase and methicillin as substrate with D_2O as solvent. Once again, a difference spectrum for the ES complex was obtained, but this time the peak was centered at 1752 $\pm$ 2 cm^{-1}, as compared to 1756 $\pm$ 2 cm^{-1} for the corresponding species in H_2O, Figure 4. This experiment was repeated several times, and invariably the ES complex peak was at an experimentally significant lower frequency in D_2O. The effect was even more prominent in the spectra prior to subtraction of the substrate, in that in D_2O, a distinct shoulder corresponding to ES was clearly visible. The direction of this shift is consistent with that expected for replacement of hydrogen bonding to H with that to D.

Monitoring the β-Lactamase Turnover Reaction by FTIR. To confirm the assignment of the ES complex peak and to ensure that there were no perturbations due to the use of the S70A mutant, we also examined the reaction of wild-type β-lactamase with a slowly hydrolyzed substrate. Cefoxitin is a very poor β-lactamase substrate, due to the presence of the 7α-methoxy group. Knowles and coworkers (*7*) previously demonstrated that its very slow rate of turnover with the wild-type *E. coli* TEM enzyme permitted the application of FTIR to monitor the catalytic reaction. In the present instance, it was chosen for these experiments on the basis

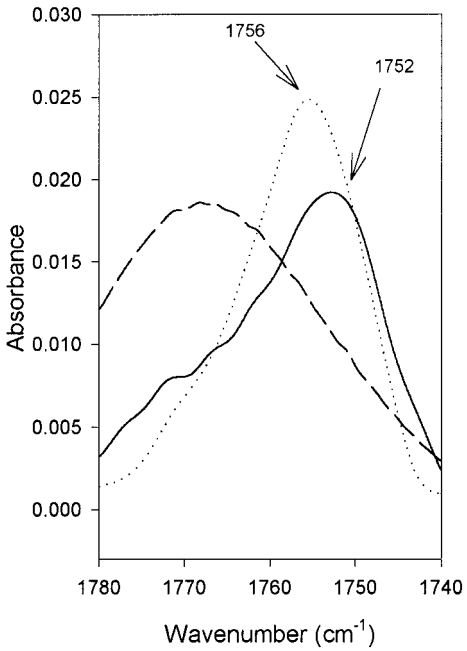


FIGURE 4: Effect of deuteration on the position of the ES complex band. The dashed line is for unbound methicillin. When bound to S70A β-lactamase in D_2O, the peak for the ES complex (solid line) is shifted to 1752 cm^{-1}, in contrast to the peak in H_2O (dotted), which is at 1756 cm^{-1}. The shift to lower frequency for the deuterated system is due to stronger hydrogen bonding of the β-lactam carbonyl in the oxyanion hole. Difference spectra for both ES complexes have been multiplied by 10 to normalize them to the equivalent concentration of the unbound substrate.

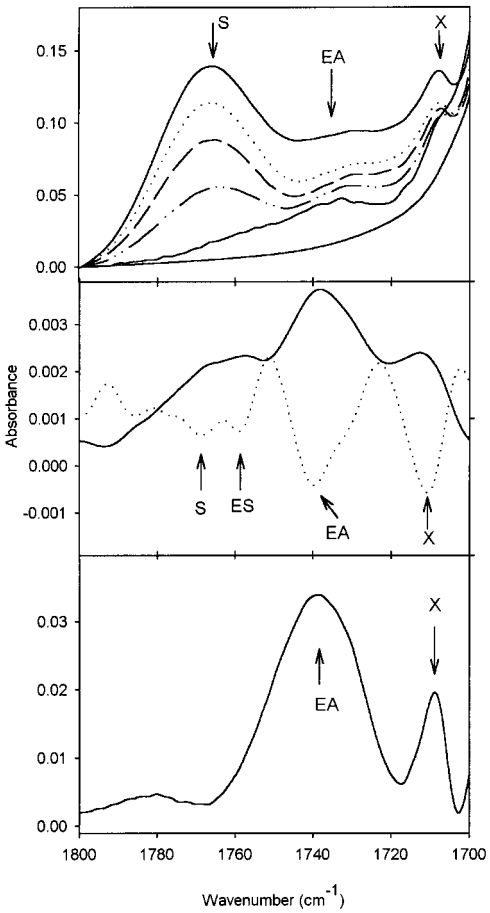


FIGURE 5: Spectral changes associated with the hydrolysis of cefoxitin by wild-type β-lactamase. Top panel: The 1800−1700 cm^{-1} region of the "raw" spectra (water, buffer, etc. subtracted), showing the substrate contribution at 1767 cm^{-1} and its time-dependent decrease. A 4-fold molar excess of cefoxitin was used in this experiment. New peaks in the vicinity of 1740 and 1710 cm^{-1} are apparent during the reaction, corresponding to the acyl−enzyme and an unidentified, enzyme-bound species, X. The top spectrum was taken at 3 min, and the lower ones were taken (in order) at 1, 2, 4, 8, and 36 h. The final spectrum shows only the enzyme contribution. The penultimate spectrum shows essentially all the substrate consumed, but a significant amount of acyl−enzyme present. Center panel: Spectrum of a mixture of 180 μM cefoxitin and 90 μM β-lactamase, after 8 h incubation at 30 °C, pH 7.0, from which the enzyme spectral contribution has been subtracted (solid line). At this stage of the reaction, almost all the substrate has been hydrolyzed. The dotted line shows the corresponding second derivative, showing four prominent features, attributed to the substrate, ES complex, acyl−enzyme, and species X, in order of decreasing frequency. Bottom panel: Full difference spectrum from an early time point {(E + S) − E − S − P}. The band at 1739 cm^{-1} is attributed to the acyl−enzyme, and that at 1709 cm^{-1} is assigned to the species X.

that it was expected to undergo very slow turnover with wild-type β-lactamase and to form a long-lived acyl−enzyme intermediate. As shown in Figure 5, we were able to monitor the catalytic reaction by using ATR-FTIR to collect the spectra of aliquots removed after various time intervals in a reaction starting with a 4-fold excess of substrate. The figure shows the concurrent loss of substrate at 1767 cm^{-1} and the steady-state presence of the enzyme-bound species in the vicinity of 1638 cm^{-1}. Detailed analysis of the new peaks due to the catalytic species, after subtraction of the contributions of the enzyme, substrate, and product revealed three components, centered at 1755, 1738, and 1709 cm^{-1} (Figure 5). The final spectrum showed no bands above 1700 cm^{-1}. The enzyme was shown to still be catalytically active at the completion of the experiments, using nitrocefin as a chromophoric substrate. Similar difference spectra, with bands at 1739 and 1709 cm^{-1}, were obtained when a 1:1 mixture of enzyme and substrate (180 μM cefoxitin) was used (Figure 5). The bands at 1755 cm^{-1} in the difference spectra are attributed to the ES complex, and those at 1738−1739 cm^{-1} are attributed to the acyl−enzyme. The source of the narrow band at 1709 cm^{-1} (marked X in Figure 5) is not clear but most likely corresponds to either a protonated carboxyl in some enzyme-bound species or an alternate conformation of the acyl−enzyme (see Discussion). This band is absent in the reactions with S70A β-lactamase.

DISCUSSION

Catalytic Activity of S70A β-Lactamase. Interestingly, even though the catalytic activity of the S70A mutant of *B. licheniformis* β-lactamase is only one-millionth that of the wild-type, the mutant still shows a large rate enhancement relative to the nonenzyme-catalyzed reaction at pH <9, because the wild-type enzyme is such a good catalyst. Of special note is the pH dependence of the reaction, which is bell-shaped like that of the wild-type, and the fact that, unlike the wild-type, the mutant undergoes significant product inhibition. The pK_1 value for the mutant is the same as that for the wild-type enzyme, whereas the pK_2 is increased by 2 units, leading to a broad plateau for the pH optimum, between pH 6 and pH 9, where k_{cat}/K_m is 25 M^{-1} s^{-1}. The simplest explanation of the bell-shaped pH dependence of the S70A β-lactamase-catalyzed reaction is that the same groups that are responsible for the pH-dependence of the

wild-type enzyme also are functional in this mutant. However, for the mutant, the catalytic mechanism must be very different, in view of the absence of the active site nucleophile. The catalytic reaction presumably involves general acid−general base catalysis by the groups whose ionizations are reflected in pK_1 and pK_2 of the S70A catalytic reaction. These are most likely Glu166, acting as a general base to activate the attacking water molecule, and Lys73/Lys234 to facilitate protonation of the lactam nitrogen leaving group (Lietz et al., in press). The FTIR data suggest that binding of the substrate to the enzyme leads to distortion of the β-lactam carbonyl: this is likely to shift the conformation of the substrate toward that of the transition state for hydrolysis. Consequently, attack by a water molecule on the carbonyl is expected to be more facile than in the absence of the enzyme. This is the probable source of the rate enhancement induced by the S70A mutant over the nonenzyme-catalyzed hydrolysis.

S70A β-Lactamase and the Michaelis Complex. By making the S70A mutant, which is unable to form an acyl−enzyme intermediate by virtue of the absence of the active site serine nucleophile, we reasoned that we should be able to make stable ES complexes with various substrates. The ATR-FTIR spectrum of the S70A/substrate complex was then used to compare the structure of the enzyme-bound versus free substrate to determine if binding to the enzyme induces distortion or polarization of the lactam carbonyl. We have focused most of our attention on the 1700−1800 cm^{-1} spectral region because there are no absorbance peaks from the enzyme or the product in this region, and this is the region where the β-lactam carbonyl stretch of the substrate occurs. The overlap of substrate, product, and enzyme components in the 1600−1700 cm^{-1} region makes assignment of observed differences in this region more challenging. The substrate β-lactam carbonyl spectrum is expected to be quite sensitive to the nature of the interaction with the enzyme as well as the progress of the catalytic reaction. For the penicillin and cephalosporin substrates we have studied, the C═O stretch frequency is in the 1765−1775 cm^{-1} region. Upon hydrolysis, the C═O frequency is shifted to well below 1700 cm^{-1}.

The FTIR analysis of the interaction of S70A β-lactamase with several penicillin and cephalosporin substrates demonstrates two important points. First, we conclude that the enzyme forms a noncovalent complex in which the critical lactam carbonyl is in essentially the same environment regardless of the substrate structure. This suggests that upon binding, the β-lactam region of the substrate is positioned identically, regardless of the preferred conformation of the free substrates and possible unfavorable steric interactions with distant parts of the substrates (e.g., the C6/7 substituents of substrates such as cefoxitin). Second, the average shift of −13 cm^{-1} observed in the ES complexes as compared to the free substrates indicates substantial ground-state strain and distortion upon binding. This is one of very few direct demonstrations of such strain.

A major source of the carbonyl strain/distortion would be expected to come from binding of the substrate forcing the β-lactam carbonyl into the "oxyanion hole" (*10*) where it interacts with the backbone amides of Ser70 and Ala237 via two hydrogen bonds. The similarity of the position of the carbonyl stretch frequency for different substrates, regardless of that in the unbound substrate, is best explained by the hypothesis that the positioning and the orientation of the lactam ring, and especially the carbonyl and nitrogen, are essentially identical for all substrates.

The magnitude of $\Delta\nu$ (13 cm^{-1}) for the substrate in the ES complex as compared to free in solution is difficult to analyze quantitatively. This is because it may reflect a combination of effects, especially hydrogen bonding, the hydrophobic/hydrophilic environment, and possible steric distortion of the sp^2-hybridized carbon and the β-lactam ring as well as local environment effects on the vibrational transition dipole of the bond itself. There are competing effects of hydrophobicity and hydrogen bonding on the IR frequency of a carbonyl stretch: increasing hydrophobicity leads to higher frequencies, whereas increased hydrogen bonding leads to lower frequencies. If the local environment is less polar than water, as is likely, then the actual distortion or polarization of the bond will be greater than it appears to be. Using a simple relationship between bond strength and frequency of vibration proposed by Wharton and co-workers (*12*), we estimate that if the only effect on the C═O frequency were due to hydrogen bonding, each hydrogen bond to the lactam carbonyl in the oxyanion hole would contribute about 3 kJ/molecule more than in the unbound state (presumably there will be some hydrogen bonding to the carbonyl by the solvent). As noted, however, likely environmental effects mean that this is a lower limit.

To confirm and attempt to quantify the oxyanion-hole-induced strain/distortion, the hydrogen bonds were replaced with deuterium, which would be expected to lower the frequency of the carbonyl due to the lower zero point energy of deuterium (*11*). Since most of the other factors contributing to the shift in frequency on binding will be unaffected by this substitution, it provides a more direct measure of the contribution of these hydrogen bonds. Using the above-mentioned relationship between $\Delta\nu$ and bond strength, we estimate the replacement of H by D leads to a change of about 0.8 kJ/mol per hydrogen bond. This value is in good agreement with previous estimates, both theoretical (*11*) and experimental (*13*−*15*). The fact that this value is similar to that expected for two hydrogen bonds suggests that the major source of the observed change in carbonyl stretch frequency is due to the hydrogen bonding to the backbone amide groups and the ensuing distortion or polarization of the carbonyl bond.

Monitoring Turnover by FTIR: Wild-Type β-Lactamase and Cefoxitin. Cefoxitin is a cephalosporin with a 7α-methoxy group, which makes it a very poor β-lactamase substrate: it is thought that the methoxy group occupies the site of the deacylating water, thus leading to rate-limiting deacylation and the accumulation of the acyl−enzyme intermediate. It was chosen for these experiments on the basis that it was expected to form a long-lived acyl−enzyme intermediate (*7*). The combination of wild-type enzyme and a slowly hydrolyzed substrate also offers the potential to determine the position of the frequencies for the β-lactam carbonyl in both the ES and the acyl-enzyme species with the wild-type enzyme. Using wild-type β-lactamase, it was possible to monitor hydrolysis of the cefoxitin as a function of time by the loss of substrate, as manifested by the decrease in substrate absorbance at 1767 cm^{-1}. With a small excess of substrate, we observed the concurrent loss of substrate

and the steady-state presence of the enzyme-bound species ES and EA. At later times in the reaction, little free substrate is present and almost all the enzyme is in the form of the acyl−enzyme. Our interpretation that the band at 1738−1739 cm^{-1} corresponds to the acyl−enzyme is based on spectra that show peaks in the vicinity of 1738 cm^{-1} for several other mutant β-lactamase substrate reactions in which the acyl−enzyme is expected to accumulate (Hokenson, M., and Fink, A. L., unpublished data). The ethyl ester of benzylpenicilloic acid has been used as a model for the β-lactamase acyl−enzyme: the carbonyl stretch in aqueous solution (D_2O) is at 1726 cm^{-1} (*12*). Thus, the position of the cefoxitin acyl−enzyme band at a higher frequency (1738 cm^{-1}) suggests that it may be in a hydrophobic environment or that the local environment affects the transition dipole coupling interactions.

There are two possible sources of the 1709 cm^{-1} band: either a protonated carboxyl in a hydrophobic environment (protonated carboxyls may have IR bands as high as 1750 cm^{-1}) or an alternate conformation of the acyl−enzyme. If the band is due to a carboxyl, it is not clear if it belongs to an enzyme-bound substrate or product or an enzyme group such as Glu166. However, it must be both a carboxyl with a high p*K* (at least in the enzyme-β-lactam species) and in a reasonably nonpolar environment to account for such a high frequency. It is also possible that it corresponds to an alternative conformation of the acyl−enzyme (*8*). The species responsible for the peak at 1709 cm^{-1} is absent in the final spectrum after all the substrate and acyl−enzyme are gone, indicating that it reflects an enzyme-bound species. Unlike the bands corresponding to the ES and EA intermediates, which are rather broad, reflecting multiple conformational substates, that at 1709 cm^{-1} is quite narrow, suggesting the substrate in this species is in a rather rigid conformational environment. As with the ES complex, the position of this band is independent of substrate structure. Such a peak does not occur if the penicilloic acid product is mixed with the enzyme, indicating that it is not a simple enzyme−product complex. Further, it is not observed in the complexes with S70A β-lactamase, suggesting that it is either an acyl−enzyme or enzyme−product complex. Further investigations are necessary to identify its nature.

Previously, Knowles and coworkers (*7*) investigated the interaction of the *E. coli* TEM β-lactamase with cefoxitin, using transmission mode FTIR, in which the enzyme concentration was >8 mM (240 mg/mL). They reported a transient peak at 1753 cm^{-1}, which they attributed to the acyl−enzyme. However, examination of their data (their Figure 3) shows the presence of a smaller band at 1738 cm^{-1}. Thus, we believe that under their experimental conditions (the *E. coli* enzyme has significantly different kinetics properties to that from *B. licheniformis*), the steady-state concentration of ES exceeded that of the acyl−enzyme, leading them to propose that the 1753 cm^{-1} component arose from the acyl−enzyme, rather than the ES complex. Recently, the IR spectra of β-lactams complexed to a class C β-lactamase and a penicillin-binding protein (PBP2x) have been reported (*8*, *12*). Transient peaks in a single-turnover experiment with the β-lactamase and methicillin at 1742, 1728, 1707, and 1683 cm^{-1} were attributed to different conformations of the acyl−enzyme (*8*). Further experiments are in progress to identify source of the peak at 1709 cm^{-1} in the catalytic turnover reaction. The results of this investigation demonstrate the value of combining site-specific mutagenesis with ATR-FTIR analysis to obtain detailed information about normally transient species in enzyme catalysis.

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