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## Accelerated Publications

# Multiple Conformations of the Acylenzyme Formed in the Hydrolysis of Methicillin by *Citrobacter freundii* β-Lactamase: a Time-Resolved FTIR Spectroscopic Study†

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ABSTRACT: Time-resolved infrared difference spectroscopy has been used to show that the carbonyl group of the acylenzyme reaction intermediate in the *Citrobacter freundii* β-lactamase-catalyzed hydrolysis of methicillin can assume at least four conformations. A single-turnover experiment shows that all four conformations decline during deacylation with essentially the same rate constant. The conformers are thus in exchange on the reaction time scale, assuming that deacylation takes place only from the conformation which is most strongly hydrogen bonded or from a more minor species not visible in these experiments. All conformers have the same (10 $cm^{-1}$) narrow bandwidth compared with a model ethyl ester in deuterium oxide (37 $cm^{-1}$) which shows that all conformers are well ordered relative to free solution. The polarity of the carbonyl group environment in the conformers varies from 'ether-like' to strongly hydrogen bonding (20 kJ/mol), presumably in the oxyanion hole of the enzyme. From the absorption intensities, it is estimated that the conformers are populated approximately proportional to the hydrogen bonding strength at the carbonyl oxygen. A change in the difference spectrum at 1628 $cm^{-1}$ consistent with a perturbation (relaxation) of protein β-sheet occurs slightly faster than deacylation. Consideration of chemical model reactions strongly suggests that neither enamine nor imine formation in the acyl group is a plausible explanation of the change seen at 1628 $cm^{-1}$. A turnover reaction supports the above conclusions and shows that the conformational relaxation occurs as the substrate is exhausted and the acylenzymes decline. The observation of multiple conformers is discussed in relation to the poor specificity of methicillin as a substrate of this β-lactamase and in terms of X-ray crystallographic structures of acylenzymes where multiple forms are not apparently observed (or modeled). Infrared spectroscopy has shown itself to be a useful method for assessment of the uniqueness of enzyme−substrate interactions in physiological turnover conditions as well as for determination of ordering, hydrogen bonding, and protein perturbation.

Many approaches have been used to gain a deeper insight into the mechanism of action of β-lactamases, which confer resistance upon bacteria against β-lactam antibiotic action (*1*). While X-ray crystallography can provide a detailed picture of the static enzyme or 'stable' reaction intermediate, infrared spectroscopy can be used to obtain time-resolved information concerning the 'working' system in close to physiological conditions (*2*). The information provided by IR measurements, in the form of hydrogen bonding, conformational distributions, and exchange, is less structurally rigorous than that provided by crystallography but fills a gap between the outright structural methods and the potentially

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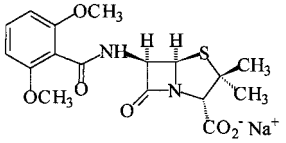
fast but structurally weak methods of UV/vis absorbance and fluorescence spectroscopies (*3*, *4*). To our knowledge, there is only a single example in the literature of the application of IR spectroscopy to the study of the $\beta$-lactamase mechanism. Thus, Knowles and co-workers (*5*) studied the interaction of the class A, plasmid-encoded *E. coli* RTEM enzyme with the cephalosporin cefoxitin. They observed the formation and subsequent decline of an absorption band characteristic of an ester carbonyl group upon acylation with the antibiotic. This was taken as evidence that catalysis of the hydrolysis of cefoxitin proceeds via an acylenzyme. On the basis of subsequent model studies, performed in our laboratory (*4*), it may be concluded that this ester carbonyl, whose absorption at 1753 $cm^{-1}$ decays over a period of several minutes, is poorly, if at all, hydrogen bonded into the oxyanion hole catalytic device. This correlates with cefoxitin having a low deacylation rate and being a very poor substrate for the enzyme with respect to turnover. In this paper we report some IR spectroscopic measurements of the *Citrobacter freudii* (AmpC) enzyme acylated with the semisynthetic penicillin methicillin (**1**). Methicillin was introduced



Methicillin (1)

for treatment of Gram-positive infections and is resistant to hydrolysis by the staphylococcal penicillinase. It is slowly hydrolyzed by the class C enzymes and therefore makes a useful probe for the mechanisms of these enzymes (*6*). Many common structural features are shared among the $\beta$-lactamases and the antibiotic target transpeptidases, which indicates that these protein families have a common ancestry (*7*, *8*). The *Citrobacter* enzyme is a class C enzyme of $M_r$ 39 000 and is chromosomally encoded. It is inducible in the presence of $\beta$-lactams, and mutants derepressed for $\beta$-lactamase synthesis can be selected in the presence of antibiotics. The structure of the enzyme acylated with aztreonam has been reported (*9*). A well-organized interaction of the aztreonam acyl carbonyl group with the oxyanion hole is present in this complex. A single conformer of the acyl group has been modeled to fit the electron density. This and other similar structures (*10*, *11*, *25*, *26*) form the basis for the discussion of the properties of the acylenzyme with methicillin.

## MATERIALS AND METHODS

*Materials.* Deuterium oxide (99%), *p*-cyanobenzaldehyde, and ethylamine hydrochloride were supplied by Aldrich, Gillingham, Dorset, U.K. All other chemicals were obtained from Sigma, U.K.

The Schiff's base formed between *p*-cyanobenzaldehyde and ethylamine by elimination of water was prepared by stirring *p*-cyanobenzaldehyde (0.1 g) with ethylamine hydrochloride (0.07 g) and triethylamine (0.1 mL) in ethanol (2 mL) for 48 h at room temperature. The crystalline product was collected by rotary evaporation and was used as such for preparation of a KBr disk from which the IR spectrum was obtained. The product showed a CN absorbance at 2229 $cm^{-1}$, a very small peak at 1709 $cm^{-1}$ from residual aldehyde, and a band at 1643 $cm^{-1}$ arising from the Schiff's base —C═N— group. The relative absorbance intensity of the imine group as compared with the aldehyde carbonyl group (ca. 30%) was calculated by using the cyano group absorbance in the parent aldehyde and Schiff's base as a reference.

The *Citrobacter freundii* (*E. coli* Amp-C type) $\beta$-lactamase was prepared and its concentration determined as previously described (*12*). For IR experiments, the enzyme (5 mL) was concentrated to 0.5 mL, washed, and reconcentrated with 3 $\times$ 5 mL of 10 mM Tris buffer, pH 7.0, using an Amicon pressure concentrator, freeze-dried, and then dissolved at 0.5 mM in 10 mM Tris buffer, pM[1] 7.25, made up in deuterium oxide. This solution was kept at 4 °C for 48−72 h prior to experiments to ensure extensive hydrogen−deuterium exchange.

*Infrared Experiments.* Methicillin was dissolved in the same buffer immediately prior to experiments. The reaction mixture for the turnover experiment, in which there was a 2.7-fold excess of methicillin over the enzyme, comprised $\beta$-lactamase (100 $\mu$L, 0.5 mM) and methicillin (10 $\mu$L, 15 mM) of which 90 $\mu$L was injected into the 'in situ' IR cuvette described previously (*4*, *13*). For the single-turnover experiment, 2 $\mu$L of methicillin solution was used to ensure that [E] $\geq$ [S]. IR spectra were measured using a Bruker IFS66 spectrometer, and 256 scans were accumulated, centered upon 30 s intervals, over a period of 20 min at 4 $cm^{-1}$ resolution and a scan rate of 17 $s^{-1}$, with a scanner modulation frequency of 180 kHz. The absence of interference by water vapor bands in the IR difference spectra was ascertained by superposition of a water vapor spectrum to show that there were no coincident bands.

## RESULTS AND DISCUSSION

*Single-Turnover Experiment.* In the single-turnover experiment shown in Figure 1, the bands assigned to the acyl carbonyl group occur at 1742, 1728, 1707, and 1683 $cm^{-1}$. There is some noise in the spectra, but kinetic analysis shows that all of these bands decline during deacylation with essentially the same first-order rate constant [$(8.3 \pm 0.9) \times 10^{-3}$ $s^{-1}$, 18 data points used for estimation of each rate constant]. This implies that the conformers are in exchange on the time scale of the experiment (*13*, *25*, *26*). Product (carboxylate) formation is represented by the band at 1593 $cm^{-1}$, and the rate constant for the formation of this band is $(1.2 \pm 0.2) \times 10^{-2}$ $s^{-1}$ (18 data points), in reasonable agreement with that for the loss of the acylenzyme species. While the assignment of the three bands at 1742, 1728, and 1707 $cm^{-1}$ is satisfactory, it is possible that the band at 1683 $cm^{-1}$ arises from perturbation of the protein structure upon acylation. This could occur in one or more $\beta$-turns in the protein or reflect a change in the minor high-frequency component of antiparallel $\beta$-sheet, since both these structural features absorb in this region (*14*, *15*). Protein perturbation bands are often complex in shape so the relatively simple Gaussian form of the band at 1683 $cm^{-1}$ argues in favor of assignment to a strongly hydrogen bonded ester carbonyl group conformer. Measurements made on substrates in free solution and model esters suggest that the absorption

[1] Abbreviation: pM, pH meter reading in deuterium oxide solution.

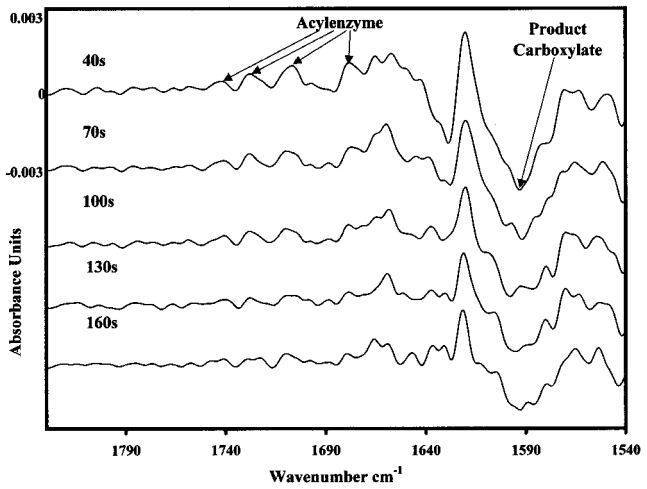



FIGURE 1: Time course of a single turnover in the *Citrobacter freudii* $\beta$-lactamase-catalyzed hydrolysis of methicillin. The first spectrum was recorded ca. 40 s after mixing of the enzyme (0.5 mM) and methicillin (0.3 mM) in 10 mM Tris buffer, pM 7.25, in deuterium oxide. Subsequent spectra were recorded at 30 s intervals as described under the Materials and Methods. Difference spectra were constructed by dividing each of the single beam spectra by the last one. A total of 19 spectra were used for kinetic analysis, and those near the end of the reaction were superimposable (subject to noise fluctuations), which showed that the reaction was complete. A subset of early spectra is shown in the Figure. Note that the unusual form of the difference spectra results from the 'first minus last' method used for their construction. Thus, all the product absorbance is present in the first difference spectrum and is a negative feature which disappears with time. The acylenzyme features, fully formed before the first spectrum was taken at 40 s, decrease with time. The absorbance scale refers to the first spectrum but is the same for all the stacked plots. Only the zero level has been shifted downward for the subsequent plots.

coefficients of the carbonyl groups in each of the conformers are likely to be similar (*4*, *16*, *17*). Therefore the distribution of the conformers can be calculated from the band areas to give the following: 1737 $cm^{-1}$, 10%; 1727 $cm^{-1}$, 20%; 1707 $cm^{-1}$, 29%; and 1683 $cm^{-1}$, 41%. There is a progression in the population of the conformers as the hydrogen bonding and/or the polarity of the carbonyl environment increases, since hydrogen bonding and an associated (or conceivably independent) increase in the polarity of the environment have the effect of lowering the carbonyl frequency (see below). The width of an IR band is inversely proportional to the mobility or dispersion of the absorbing species, and it has generally been observed that 'productively' bound, strongly hydrogen bonded conformers show narrow IR bands compared with a model compound in free aqueous solution (*4*, *16*). Unexpectedly, the four bands assigned to acylenzyme conformers here all have similar very narrow bandwidths of ca. 10 $cm^{-1}$, which implies a high degree of ordering and lack of mobility in all conformers, when compared with the bandwidth of the model compound ethyl benzylpenicillinoate in deuterium oxide of 37 $cm^{-1}$ (*4*). We cannot rule out the existence of conformers with even lower frequencies and that are even more strongly hydrogen bonded, since there is a distinct but complex feature centered around 1660 $cm^{-1}$. This is close to the region where the $\alpha$-helix absorbs, and this absorbance might thus arise from a relatively minor distortion of $\alpha$-helix.

As mentioned above, the possibility exists that the frequency shifts arise from differences in polarity rather than

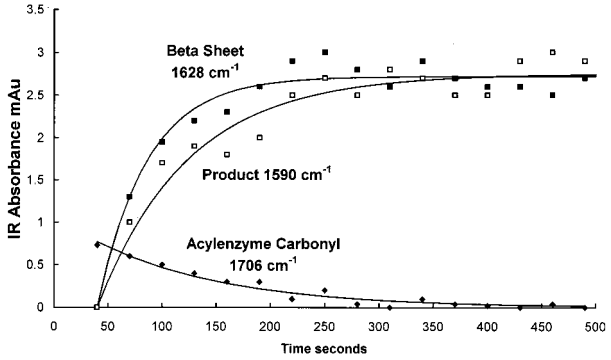



FIGURE 2: Time dependence and identities of some of the spectral changes seen in Figure 1. The lines are calculated from the rate constants given in the text.

hydrogen bonding in the enzyme-bound environment. However, since ethyl ester model of the acylenzyme absorbs at 1727 $cm^{-1}$ in deuterium oxide, the bands at 1707 and 1683 $cm^{-1}$ would have to represent conformers with carbonyl groups in environments *very* much more polar than water. Approximate estimates of the microenvironmental dielectric constants for the carbonyl groups of these conformers from IR studies of model esters gives values of 110 and 180, respectively (*17*). While not impossible, since formamide has a value of 110, these high values are more likely to arise primarily from hydrogen bonding.

The time courses of several of the dynamically changing features seen in Figure 1 are plotted in Figure 2. It is apparent that the change seen at 1628 $cm^{-1}$ proceeds at a similar but slightly faster rate [$k_1 = (2.0 \pm 0.2) \times 10^{-2}$ $s^{-1}$] compared to that of product formation. This frequency is within the range for a change in structure of protein $\beta$-sheet (1620−1640 $cm^{-1}$), and therefore the change may be ascribed to a relaxation of the structure coincident upon or immediately prior to deacylation (*14*, *15*). The observation of relaxation upon deacylation requires that a structural change of the same form is induced upon acylation but that it is complete, as is acylation (since there is no absorbance at 1760 $cm^{-1}$ characteristic of the $\beta$-lactam ring), in the first difference spectrum measured at 40 s. There is much kinetic evidence for protein structural isomerization of class C $\beta$-lactamases as a consequence of acylation and turnover; indeed, the approach to the steady state in the hydrolysis of furoylacryloylpenicillin by the *freundii* enzyme shows double-exponential behavior (*12*). The kinetic model which fits these data requires three interconvertible enzyme forms. Despite the above, it is not possible to categorically assign the change seen at 1628 $cm^{-1}$ to a change in $\beta$-sheet for the reasons given below. First, the absorbance change is not sufficiently large to be able to exclude a change in the ligand rather than the protein structure as being responsible for this feature. Thus, on acylation by benzylpenicillin, the *Strep. pneumoniae* transpeptidase PBP2x IR spectrum shows a large change at 1640 $cm^{-1}$. The intensity of the change is much larger than the intensity changes associated with the substrate and acylenzyme and so could not be explained by a change in the ligand structure. It is thus necessary to consider what changes might occur in the methicillin ester ligand of the *freundii* acylenzyme. It has been observed that absorbance develops at 280 nm in the UV spectrum during turnover in class C $\beta$-lactamase catalysis (piperacillin: *E. coli*, AmpC; M. G. P. Page, unpublished observations). When the substrate

is exhausted and the enzyme deacylates, the absorbance at 280 nm disappears. The formation of absorbance at 280 nm could represent development of a (C5−C6) enamine (>C═C−N<) with consequent thiazolidine ring opening and thiolate formation. Such behavior has been observed in the alkoxide ion catalyzed hydrolysis of benzylpenicillin, where the enamine is formed from the ester intermediate as a transient side product that reconverts to the ester and hence the acidic product as the reaction reaches completion (*18*, *19*). At the conclusion of the reaction, there is no residual enamine. Aliphatic enamines, such as would be formed from methicillin, absorb strongly in the IR with a frequency near 1655 $cm^{-1}$ (*20*) and hence might be a candidate for the change at 1628 $cm^{-1}$. The enamine absorption frequency is rather high but might be lowered in the enzyme active site environment. The change in absorption at 1628 $cm^{-1}$ represents an increase toward the end of the reaction, not a decrease as seen for the change at 280 nm in the AmpC−catalyzed hydrolysis of piperacillin or in the alkoxide ion-catalyzed hydrolysis of benzylpenicillin. As far as we are aware, there is no precedent for the formation of enamine as a persistent product, and on this basis, we conclude that this is not a viable explanation.

Another potential candidate is the imine group (−C═N−), which absorbs in the same region. This grouping forms in the tautomerization of the enamine but is generally less favorable energetically in solution chemistry than is the enamine (*20*). The imine group of the unprotonated Schiff's base prepared from *p*-cyanobenzaldehyde and ethylamine has an absorbance at 1643 $cm^{-1}$. This frequency is again significantly higher than the spectral change observed at 1628 $cm^{-1}$. The methicillin-derived imine would not be expected to absorb at 280 nm or to accumulate as a product since thiazolidine ring closure should be a rapid intramolecular addition process. The iminium form is not a candidate as the p$K_a$ is near 5.0, well below the pM of the experiment (*21*).

We conclude on the basis of the above that the observed spectral change at 1628 $cm^{-1}$ and that seen at 280 nm in the *E. coli* AmpC experiment are most readily explained in terms of a conformational change of the enzyme. The perturbation, as we have proposed for *Strep. Pneumoniae* PBP2x (*4*), is likely to be in the five-stranded $\beta$-sheet region close to the active site, sited at the junction of the $\beta$-sheet and α-helical domains of the protein, that is conserved among $\beta$-lactamases, transpeptidases, and carboxypeptidases (*7*) and that it relaxes somewhat faster than deacylation. This suggests that conformational relaxation is a prerequisite for deacylation, although the kinetic data presented here are too sparse to provide rigorous support for this hypothesis. Mobashery and co-workers have used time-resolved CD spectroscopy to show that reciprocal conformational changes occur upon acylation and deacylation of the *E. coli* TEM1, class A $\beta$-lactamase during catalysis of the hydrolysis of imipenem (*22*) and cefepime (*23*). Such conformational changes may thus be a general feature of $\beta$-lactamase-catalyzed hydrolysis of poor substrates.

*Turnover Experiments in the Presence of Excess Methicillin.* The IR difference spectra showing the time course of the turnover of a 2.7-fold excess of methicillin is shown in Figure 3, and the time dependence of the various components is shown in Figure 4. The prominent positive absorption at

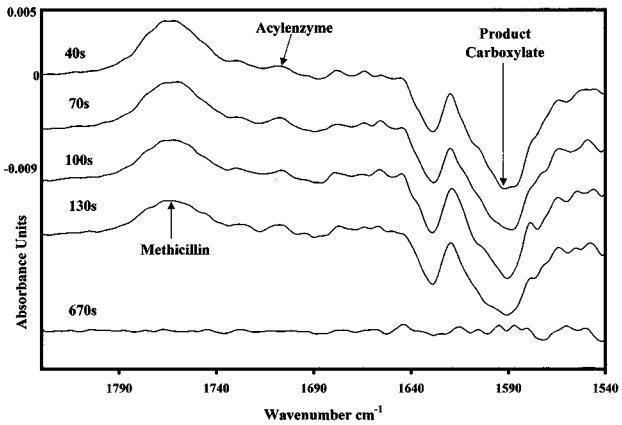



FIGURE 3: Difference spectra, acquired as in Figure 1, for 2.7 turnovers of the *Citrobacter freundii* $\beta$-lactamase-catalyzed hydrolysis of methicillin. The enzyme (0.5 mM) was mixed with methicillin (1.36 mM) ca. 40 s before the first spectrum was measured, with subsequent spectra being acquired at 30 s intervals. Other details as in Figure 1.

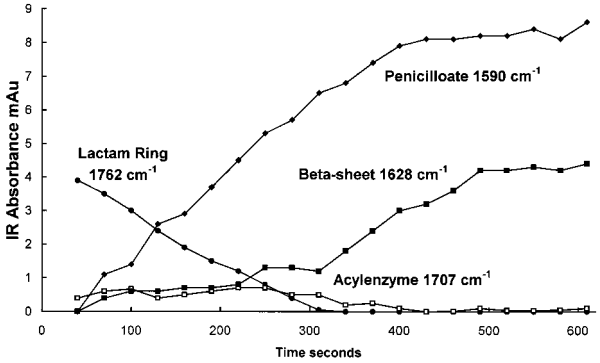



FIGURE 4: Time dependence of some of the spectral changes seen in Figure 3. Note that only the acylenzyme carbonyl band at 1706 $cm^{-1}$ is plotted. The other bands, although visible, are influenced by the lactam absorbance or are subject to a noise level which renders them unsuitable for kinetic analysis.

1762 $cm^{-1}$ represents the $\beta$-lactam carbonyl, while that at ca. 1593 $cm^{-1}$ represents the formation of the carboxylate product upon deacylation. The changes in these bands, as turnover proceeds, show the predicted behavior, i.e., a nearly linear loss of methicillin and accumulation of product respectively until the antibiotic is exhausted. Near-linear behavior is expected on the basis of the reported Michaelis parameters [$K_m$ = 2 nM, $k_{cat}$ = 0.01 $s^{-1}$, (*6*)], since the enzyme would be saturated until virtual completion of the reaction. The acylenzyme concentrations remain essentially constant until the substrate is exhausted and then decay with effectively the same first-order rate constant as in the single-turnover reaction [(9.1 ± 1.0) × $10^{-3}$ $s^{-1}$]. Although the $k_{cat}$ value measured here is in good agreement with the literature value, the $K_m$ value seems very low by comparison with literature values for many $\beta$-lactamases and a wide range of substrates. Accordingly, we have measured the $K_m$ value for methicillin using the method of reference *6* and obtain a value of 490 ± 40 nM. Although this value is much larger than the literature value, it does not negate the deductions made above and implies that acylation is very much faster than deacylation (*3*).

The band at ca. 1630 $cm^{-1}$ is, as in the single turnover experiment, tentatively assigned to a perturbation in the $\beta$-sheet structure of the enzyme. This also changes only near

the end of the overall reaction and is apparently maintained as long as there is excess substrate by rapid reacylation. In turnover conditions, the steady-state concentration of the perturbed intermediate remains constant.

*Hydrogen Bond Strength in the Acyl Group Conformers.* Ester carbonyl groups absorb at relatively high frequency [e.g., 1742 $cm^{-1}$ for ethyl benzylpenicillinoate in diethyl ether (*4*)] in apolar solvents, but hydrogen bonding or a polar environment lowers the frequency to an extent that depends on the strength of hydrogen bonding or degree of polarity. The ester carbonyl (Figure 1) which absorbs with the highest frequency at 1742 $cm^{-1}$ represents a conformer in which the carbonyl group is apparently in an environment that is well ordered (narrow bandwidth, see above) but much less polar compared with water. The carbonyl group must thus be buried in the protein and experience no hydrogen bonding in an environment with a dielectric constant similar to diethyl ether ($D = 4$). The conformer that absorbs at 1728 $cm^{-1}$ has the same frequency as the model ester in deuterium oxide, and so the carbonyl may be exposed to aqueous solvent. It is not properly aligned in the oxyanion hole and represents another nonproductive conformer (*16*, *17*). The conformer at 1707 $cm^{-1}$ shows significant hydrogen bonding, presumably but not necessarily in the oxyanion hole; it again cannot be aligned optimally. The strength of the hydrogen bonding, calculated from the frequency shift, using the Morse potential (*4*), is ca. 12 kJ/mol, capable of providing a modest rate acceleration of ca. 100-fold, if it was correctly oriented (*17*). The putative conformer which absorbs at 1683 $cm^{-1}$ experiences strong hydrogen bonding of ca. 20 kJ/mol, and we might expect this to be the 'productive' conformer for entry into the deacylation reaction channel. Hydrogen bonding of this magnitude would provide rate acceleration of 3000-fold if the strain induced in the ground state is directed to transition state formation. We cannot rule out the presence of other conformers with even stronger hydrogen bonding. The absorbance of such conformers may be obscured by the protein perturbations, or, more intriguingly, they may be sparsely populated and so unobservable, yet highly reactive.

*Why Several Conformations?* The X-ray structure of the *freundii* $\beta$-lactamase−aztreonam complex is modeled to show a single conformation of the aztreonam acyl group, as are almost all similar determinations of protein−ligand complexes by crystallography (*9*). This unimodal binding model may represent the situation in solution but may also be a consequence of crystallization or the manner in which electron density is interpreted in X-ray diffraction maps. We must ask whether the various potential conformers collapse into the most thermodynamically stable form, with good insertion in the oxyanion hole, in the crystals as is seen in the TEM1 acylenzyme formed with 6α-(hydroxymethyl)-penicillanate *(26)*. Does crystallization lead to the formation of a single thermodynamic well? Alternatively, it may be that crystallographers model only the predominant conformer and cannot get a clear picture of lesser populated ones. Only when multiple conformers are well populated is it possible to observe and model them (see, e.g., *24*). In our recent studies of a transpeptidase complex with benzylpenicillin and other antibiotics, we have observed that the acyl group of the natural benzylpenicillin antibiotic binds in a single well-defined conformation. In contrast, the semisynthetic cloxacillin shows two conformers: one reasonably well-organized and moderately hydrogen bonded and one where the carbonyl group is apparently exposed to solvent (*4*). The most likely reason for the observation of multiple conformers in the methicillin acylenzyme is the lack of specificity and hence lack of fit in the active site of the substrate, for which, as far as deacylation is concerned, it was indeed designed to frustrate $\beta$-lactamases! The second-order constant for acylation by methicillin is a healthy $5.0 \times 10^6$ $M^{-1}$ $s^{-1}$, presumably driven in large part by the intrinsic chemical energy of the lactam ring-opening process, while the deacylation rate constant is a paltry 0.01 $s^{-1}$ (*6*). For benzylpenicillin, the acylation rate constant is near-diffusion-limited at $75 \times 10^6$ $M^{-1}$ $s^{-1}$, while the deacylation constant is 31 $s^{-1}$, some 3000-fold faster than with methicillin. Unfortunately, since the reaction is so fast, we are not able to perform the obvious experiment, namely, to measure the equivalent spectra with benzylpenicillin as substrate. We conclude, then, that the enzyme is simply not able to correctly position the methicillin framework, with its bulky side chain, into a properly productive conformation with water correctly positioned for deacylation. Because it cannot achieve this, the energy barriers for acquisition of other conformations are readily surmountable: the acyl group 'flops about' in the active site. Interestingly, Mobashery, Samama, and coworkers have predicted, on the basis of X-ray crystallographic structures and molecular dynamics simulations, that two conformers of the acyl group are in rapid exchange in acylenzymes of imipenem (*25*) and 6α-(hydroxymethyl)-penicillanate (*26*) with TEM1. In each case only a single conformer is seen in the crystallographic structures. We trust that we have demonstrated in this communication how IR spectroscopy can add an extra dimension to crystallographic and computational studies by observation of the 'working machine' in solution.

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