Direct n.m.r. evidence for substrate-induced conformational changes in a β-lactamase

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Cefoxitin and other β-lactam antibiotics with a methoxy group on the α-face behave as very poor substrates of the *Bacillus licheniformis* β-lactamase. The kinetic properties of the enzyme–cefoxitin system made it theoretically suitable for a detailed structural study of the acyl-enzyme. Unfortunately, soaking the crystals in cefoxitin solution did not allow detection of a crystalline acyl-enzyme complex. In contrast, direct observation by n.m.r. of the stable acyl-enzyme formed with cefoxitin and moxalactam indicated clear modifications of the enzyme structure, which were reflected in the aromatic and high-field methyl regions of the spectrum. The return to the initial free enzyme spectrum was concomitant with the hydrolysis of the acyl-enzyme, the process being slow enough to allow multidimensional n.m.r. experiments.

INTRODUCTION

At the present time, only X-ray crystallography and multidimensional n.m.r. can be used to determine the detailed structures of enzymes and enzyme–substrate intermediates. The study of the latter, however, requires formation of rather stable complexes with half-lives ideally longer than 15 min for n.m.r. and several hours for crystallographic analysis. In addition, the enzymes must be available in large quantities, especially for the n.m.r. work, and must be sufficiently stable.

Four classes of β-lactamases, A, B, C and D, have been described. The class-A enzymes are often produced by pathogenic strains and are widely encountered in the bacterial world. Four members of this class, the TEM1, *Staphylococcus aureus* PC1, *Bacillus licheniformis* and *Streptomyces albus* G β-lactamases, have been successfully crystallized. The structures of the free enzymes have been determined to high resolution.

They usually interact with their substrates according to the three-step mechanism described by model 1:

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EC \xrightarrow{k_{+2}} EC^* \xrightarrow{k_{+3}} E + P$$

where E is the enzyme, C a substrate, EC is the Henri–Michaelis complex and EC* the acyl-enzyme (Waley, 1992). The dissociation constant of EC is $K = k_{-1}/k_{+1}$.

Depending on the relative values of k_{+2} and k_{+3}, either the Henri–Michaelis (EC) complex or the acyl-enzyme (or both) can accumulate at the steady state. A low k_{+2} value might allow the observation of the first complex and a low k_{+3}, combined with a high k_{+2}/k_{+3} ratio, that of the acyl-enzyme. Whatever the situation, the K_m value must be such that saturation of the enzyme occurs at a reasonably low substrate concentration, and, in consequence, a maximum K_m value of 1 mM appears to be a practical limit.

Several mechanism-based inactivators of β-lactamases are known [see for instance the review by Pratt (1992)] but these compounds undergo important secondary modifications after the formation of the acyl-enzyme and, in consequence, the stable adducts that can be isolated are not representative of the intermediates on the catalytic pathway (Cohen and Pratt, 1980; Fisher et al., 1980; Frère et al., 1982).

A survey of the kinetic parameters of poor substrates of the four crystallized enzymes failed to reveal any compound with which a suitably stable acyl-enzyme might accumulate. In a parallel study (Matagne et al., 1993), penicillins and cephalosporins bearing a methoxy side chain on C-6 or C-7 respectively were found to form stable acyl-enzymes with the *Actinomadura* R39 β-lactamase. Unfortunately, this enzyme is not available in large quantities and has not been crystallized.

The adduct formed on acylation of the TEM2 β-lactamase by cefoxitin can readily be isolated (Fisher et al., 1980), but it is still too unstable ($k_{+3} = 4.8 \times 10^{-3}$ s^{-1}) to allow a detailed study by X-ray crystallography or multidimensional n.m.r.

In the present paper, we have analysed interactions between various 'β-lactamase-stable' compounds and the *B. licheniformis* enzyme. The acyl-enzyme formed with cefoxitin appeared to exhibit the required characteristics for a detailed n.m.r. study.

MATERIALS AND METHODS

Chemicals, enzymes and buffers

Cefoxitin and [^{14}C]cefoxitin were obtained from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.), moxalactam, cephalexin, cephalothin and cefaclor were from Lilly Research Laboratories (Indianapolis, IN, U.S.A.), temocillin was from Beecham Pharmaceuticals (Brentford, Middx., U.K.), 7-aminodeacetoxycephalosporanic acid (7-ADCA) was from Gist-Brocades (Delft, The Netherlands), 7β-formamidoyl-7α-methoxycephalosporanic acid (FMCA) was from Hoechst

Abbreviations used: 7-ADCA, 7-aminodeacetoxycephalosporanic acid; FMCA, 7β-formamidoyl-7α-methoxycephalosporanic acid; Dnp-7ADCA, dinitrophenyl-7-aminodeacetoxycephalosporanic acid; 7-ACA, 7-aminocephalosporanic acid.

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Aktiengesellschaft (Frankfurt, Germany) and ceftazidime was from Glaxo Group Research (Greenford, Middx., U.K.) All these compounds were kindly donated by the respective companies. Dinitrophenyl-7-aminodeacetoxycephalosporanic acid (Dnp-7ADCA) was a gift from Dr. S. G. Waley, University of Oxford, Oxford, U.K. Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.) and 7-aminocephalosporanic acid (7-ACA) was from Janssen Pharmaceutica (Beerse, Belgium). The detailed structures of most of these compounds can be found in Matagne et al. (1990, 1993).

The β-lactamases of *B. licheniformis* and *Enterobacter cloacae* 908R were purified as described by Matagne et al. (1990). For the former all experiments were performed in 50 mM sodium phosphate buffer, pH 7.0, but for n.m.r., $Na_2{}^2HPO_4$ and $Na^2H_2PO_4$ were used.

Kinetic measurements

Spectrophotometric readings were performed using a Beckman DU-8 spectrophotometer connected to an Apple II microcomputer (De Meester et al., 1987). For the *B. licheniformis* enzyme, inactivation rate constants, k_i, were determined using nitrocefin ($K_m = 40\ \mu M$, $k_{cat.} = 600\ s^{-1}$) or ceftazidime ($K_m = 1.4$ mM, $k_{cat.} = 15\ s^{-1}$) as reporter substrates. The enzyme was added to 500 μl of a solution containing both the reporter substrate and increasing concentrations of cefoxitin. The values of k_i were deduced from the time-dependent decrease in reporter-substrate utilization (De Meester et al., 1987). As

$$k_i = k_{+3} + \frac{k_{+2}[C]}{[C] + K\{1 + ([S]/K_{m,S})\}} \quad (1)$$

(where [S] and $K_{m,S}$ are the concentration and K_m value of the reporter substrate), a direct proportionality between k_i and [C] indicates that $[C] \ll K\{1 + ([S]/K_{m,S})\}$ and the slope of the line yields $k_{+2}/K\{1 + ([S]/K_{m,S})\}$. If, in addition, the k_{+3} value is much smaller than that of the second term in eqn. (1), $k_{+2}[C]$ becomes the important parameter for determining the acylation rate. In re-activation experiments, the assay solution consisted of 400 μl of 100 μM cephalexin or nitrocefin. Hydrolysis of temocillin was monitored at 230 nm and that of cephalosporins at 260 nm. The steady-state parameters $k_{cat.}$ and K_m were usually determined by analysing complete reaction time courses. In some cases, K_m values were determined as K_i values with nitrocefin as substrate. In these cases, $k_{cat.}$ values were derived from initial-rate measurements at $[C] = K_m$ or $[C] \gg K_m$. Curve fitting was generally performed using the Enzfitter program (Leatherbarrow, 1987), but for the unsolved differential equations, a program written by Holzhütter and Colosimo (1990) was utilized.

Cefoxitin and its hydrolysis products were separated on a reversed-phase C2–C18 Pep-RPC HR5/5 column (Pharmacia, Uppsala, Sweden). Buffer A was 10 mM sodium phosphate, pH 6.5, and a linear gradient of acetonitrile was applied. Cefoxitin was eluted at 28 % acetonitrile and its primary hydrolysis product at 4.5 %.

C.d. and n.m.r. experiments

C.d. spectra were recorded on a Jobin-Yvon Mark V spectrophotometer at 20 °C in 10 mM sodium phosphate buffer at pH 7.0.

^{1}H-n.m.r. spectra were recorded at a frequency of 400 MHz on a Brucker AM400 spectrometer with a 5 mm double-frequency $^1H/^{19}F$ probe. The sample temperature was 30 °C. The free-induction decays were accumulated in 2048 or 4096 data points over a spectral width of 4800 Hz, with a 30° pulse (2 μs) and a relaxation time of 1–1.5 s. The residual-water signal was suppressed by applying a pulse at the appropriate frequency for 1–1.5 s (Campbell et al., 1974). Before Fourier transformation, the data were zero-filled and multiplied by a window function (gaussian). Chemical shifts were measured relative to 2,2-dimethyl-2-silapentane 5-sulphonate.

The purified protein was dialysed against 50 mM sodium phosphate buffer, pH 7.5, freeze-dried and redissolved in 450 μl of ^2H_2O. Cefoxitin dissolved in 20 μl of ^2H_2O was added to the enzyme to obtain the desired final concentration. The enzyme concentration was 1–2 mM. The pH was measured by a direct pH-meter reading without correction for the isotope effect on the glass electrode.

Isolation and quantification of the acyl-enzyme

When [^{14}C]cefoxitin was used, samples were filtered through a small Sephadex G-25 (NAP5) column equilibrated with 5 mM $Na^2H_2PO_4/Na_2{}^2HPO_4$ buffer at pH* 7.0. Fractions of volume 200 μl were collected and A_{280} and radioactivity were determined in each to give an estimation of the extent of enzyme labelling. After each filtration, the three fractions containing the highest protein concentrations were pooled and n.m.r. spectra were recorded.

Experiments with crystals

Crystals of *B. licheniformis* β-lactamase were grown as described by Dideberg et al. (1985). Binding assays were carried out by soaking the crystals in 50 or 200 mM cefoxitin dissolved in 50 mM sodium cacodylate buffer at pH 5.5 containing 10 mM NaN_3 and 15 % poly(ethylene glycol) 6000.

RESULTS

Kinetic analysis of *B. licheniformis* β-lactamase

Several substrates have been previously studied (Matagne et al., 1990), but of these, only 7-ACA and 7-ADCA exhibited relatively low $k_{cat.}$ values. Table 1 summarizes the results obtained with a series of other substrates. None exhibited a $k_{cat.}$ value significantly

Table 1 Kinetic parameters of various substrates with the *B. licheniformis* β-lactamase

The $k_{cat.}$ value represents the turnover number under saturating conditions. The errors represent S.D. values determined on the basis of the best four independent experiments.

	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
Ticarcillin*	220 ± 10	46 ± 2	4700 ± 400
Temocillin	(1.2 ± 0.5) × 10^{-3}	570 ± 30	(2.3 ± 0.5) × 10^{-3}
Cefaclor	41 ± 2	120 ± 3	400 ± 10
7-ADCA*	(2 ± 0.2) × 10^{-3}	2200 ± 200	(0.8 ± 0.07) × 10^{-3}
7-ACA*	(70 ± 4) × 10^{-3}	220 ± 20	0.33 ± 0.03
Dnp-7ADCA†	(4.3 ± 0.5) × 10^{-3}	580 ± 20	(7 ± 0.6) × 10^{-3}
Moxalactam†	(0.9 ± 0.1) × 10^{-3}	290 ± 20	(3 ± 0.4) × 10^{-3}
FMCA†	(7 ± 0.4) × 10^{-3}	560 ± 30	(10 ± 1.0) × 10^{-3}
Cefoxitin‡	(0.13 ± 0.01) × 10^{-3}	6.5 ± 2	(20 ± 5) × 10^{-3}
Cephalothin*	48 ± 2	20 ± 1	2500 ± 300

* From Matagne et al. (1990).

† K_m determined as a K_i with nitrocefin as a substrate and $k_{cat.}$ estimated as $2 \times v_{K_m}/E_0$ where v_{K_m} is the initial rate measured at $[C] = K_m$.

‡ Average of the various values given in the text and in the figure legends. K_m computed from the $k_{cat.}$ and k_{+2}/K ($= k_{cat.}/K_m$) values.

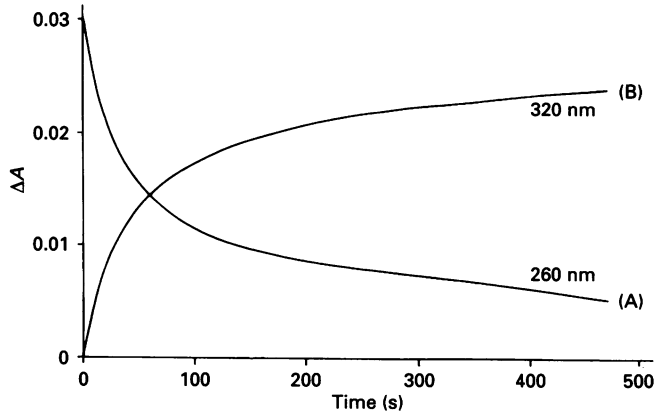


Figure 1 Hydrolysis of cefoxitin (1.78 mM) by the *B. licheniformis* β-lactamase (49 μM)

The reaction volume was 450 μl. At 260 nm (A), the optical light path of the cell was 1 mm, and at 320 nm (B) it was 10 mm. From the two curves, pseudo-first-order rate constants of 0.026 (A) and 0.020 (B) s^{-1} were computed for the substrate-induced inactivation phenomenon.

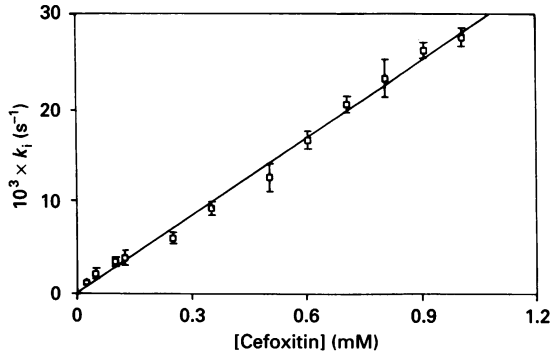
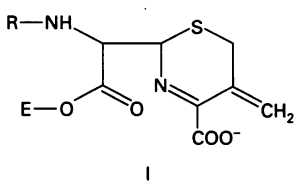


Figure 2 Variation of the apparent first-order inactivation rate constant (k_i) with cefoxitin concentration

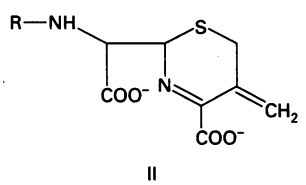
The reporter substrate was ceftazidime (50–125 μM). The slope of the line yielded $k_{+2}/K = 27.4 \pm 0.1$ $M^{-1} \cdot s^{-1}$ because, for ceftazidime, the K_m value was much larger than [S] [see eqn. (1)].

lower than 10^{-3} s^{-1}. However, preliminary experiments performed with cefoxitin indicated a very low rate of hydrolysis ($k_{cat.} < 5 \times 10^{-4}$ s^{-1}) and an apparent K_m value lower than 10 μM (measured as K_i in a competition experiment with nitrocefin). After a 60 min inactivation of 10 μM enzyme by 250 μM cefoxitin, the mixture was diluted 1000-fold and the rate of activity recovery was characterized by a first-order rate constant of $(1.35 \pm 0.1) \times 10^{-4}$ s^{-1}. Formation of the stable intermediate could be followed at both 260 and 320 nm (Figure 1) and exhibited the characteristic features of a burst, the size of which corresponded to $(0.97 \pm 0.05)E_0$ where E_0 was the total enzyme concentration. The absorbance decrease at 260 nm reflects the hydrolysis of the β-lactam amide bond ($\Delta_\varepsilon = -6000$ $M^{-1} \cdot cm^{-1}$ for cefoxitin) and the increase at 320 nm is due to the formation of the conjugated double-bond system resulting from elimination of the carbamate moiety on C-3′ ($\Delta_\varepsilon = 600$ $M^{-1} \cdot cm^{-1}$). These results were in agreement with the simple three-step model 1 with $k_{+3} \ll k_{+2}$. Moreover, the increase in absorbance at 320 nm was concomitant with the decrease at 260 nm, which indicated that elimination of the carbamate moiety on C-3′ was simultaneous with or faster than the opening of the β-lactam amide, thus yielding the acyl-enzyme I as the sole covalent intermediate.

The rate of formation of intermediate I (see structures I and II below) was further studied with the help of ceftazidime as a reporter substrate. The results, displayed in Figure 2, and obtained at cefoxitin concentrations ranging from 25 μM to 1 mM, indicated a k_{+2}/K value of 27.4 ± 0.1 $M^{-1} \cdot s^{-1}$ with k_{+2} and K values larger than 0.03 s^{-1} and 1 mM respectively. A similar experiment performed with nitrocefin as the reporter substrate yielded a similar k_{+2}/K value (22 ± 3 $M^{-1} \cdot s^{-1}$). These values were in fair agreement with those that could be deduced from the k_i values of Figure 1 (14 ± 3 $M^{-1} \cdot s^{-1}$) assuming that $k_i = (k_{+2}/K)$ [C].



I



II

However, the initial product II was found to be unstable. Its disappearance could be followed by n.m.r. (disappearance of the resonances at 5.64 and 5.68 p.p.m.), h.p.l.c. (disappearance of the initial product eluted at 4.5% acetonitrile) or u.v. spectroscopy (decrease in A_{320}). These three methods yielded a first-order rate constant of $(3 \pm 1) \times 10^{-5}$ s^{-1} for the degradation of product II. This degradation was also accompanied by a decrease in A_{260}, but a Δ_ε at 260 nm of -280 $M^{-1} \cdot cm^{-1}$ was found which remained negligible when compared with that observed for the first hydrolytic reaction (-6000 $M^{-1} \cdot cm^{-1}$), and including the degradation of product II in the analysis of the curves presented in Figure 1 did not significantly modify the results. It was also verified that product II, as obtained with the help of the *E. cloacae* 908R β-lactamase [$k_{cat.} = 0.08$ s^{-1} (Galleni et al., 1988)], failed to inhibit the *B. licheniformis* enzyme at concentrations up to 6.7 mM.

Finally, the use of ^{14}C-labelled cefoxitin allowed the proportions of active and acylated enzyme to be monitored. As shown in Figure 3, and in agreement with the measured k_{+2}/K value, nearly 100% of the enzyme was acylated within a few minutes. Subsequently, slow recovery of activity was concomitant with the loss of label from the enzyme. Figure 3 also shows the n.m.r. high-field methyl-group domain for four samples of protein isolated after increasing periods of time (see below for further details).

C.d. spectra and crystallographic studies

The c.d. spectrum of the free enzyme (1 mg/ml, i.e. 33 μM) recorded between 210 and 300 nm was superimposable on those of duplicate samples of enzyme that had been added with 100 μM and 1 mM cefoxitin and left at 30 °C for 30 min, conditions under which acylation was essentially complete.

When crystals were soaked in 50 mM cefoxitin, no modifications were observed in the diffraction pattern. Conversely, in 200 mM cefoxitin, superficial dissolution of the crystals occurred and the diffraction properties were lost.

N.m.r. experiments

After formation of adduct I, the ^1H-n.m.r. spectrum of the enzyme was modified. Although several complex modifications were observed in the aromatic region of the spectrum, the most

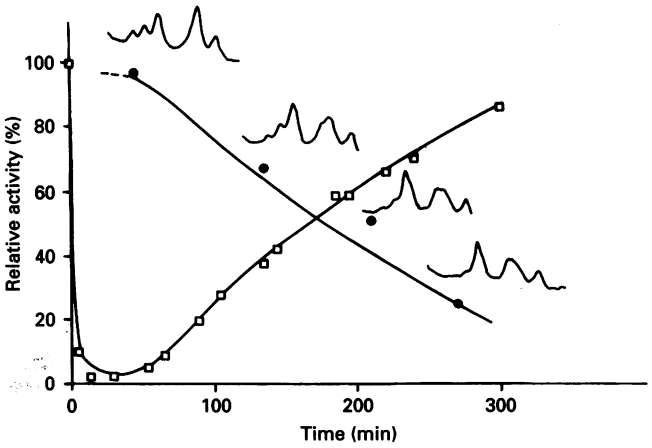


Figure 3 Simultaneous monitoring of enzyme activity, protein-bound [^{14}C]cefoxitin and n.m.r. spectra

The enzyme (0.85 mM) was incubated with [^{14}C]cefoxitin (1.2 mM), and, after various periods of time, the following measurements were performed: (1) activity (□) against cephalexin was determined in 10 μl samples after a 90-fold dilution. The fitting procedure (solid curve) yielded $k_{+2}/K = 15.5 \pm 5\ M^{-1} \cdot s^{-1}$ and $k_{+3} = (0.117 \pm 0.001) \times 10^{-3}\ s^{-1}$; (2) molar ratio of bound [[^{14}C]cefoxitin]/[enzyme] (in %) was determined after filtration of a 400 μl sample through Sephadex G-25 (●); (3) high-field methyl domain of the n.m.r. spectrum was recorded for each of these samples. More detailed spectra are shown in Figure 5.

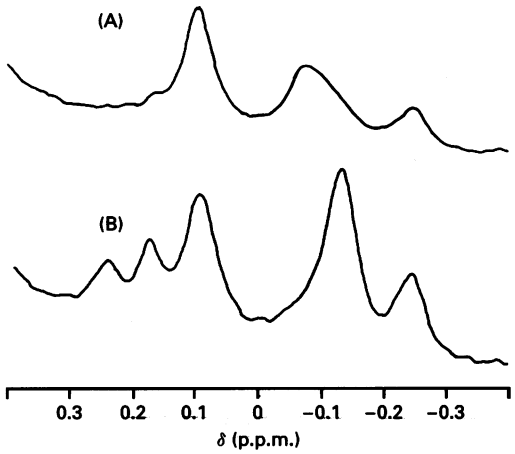


Figure 4 N.m.r. spectra of the *B. licheniformis* enzyme in the high-field methyl area

(A) Free enzyme (0.85 mM); (B) enzyme at the same concentration in the presence of 1.2 mM cefoxitin. The spectra were recorded at 30 °C and pH* 7.0 15–20 min after mixing of the reagents. Note that the spectra of cefoxitin and its hydrolysis product II do not exhibit any resonance below 3.3 p.p.m.

striking were those in the high-field methyl-group area around 0 p.p.m. (Figure 4). The modifications were entirely reversible and the spectrum returned to its initial shape as the acyl-enzyme disappeared (Figure 5), a phenomenon that could be roughly correlated with the recovery of activity (Figure 3). To obtain a more quantitative assessment and despite the sometimes erratic baselines, the resonances between 0.3 and 0.1 p.p.m. and at 7.43 p.p.m. (not shown) were estimated by planimetry, which yielded first-order rate constants of about 1.0×10^{-4} and

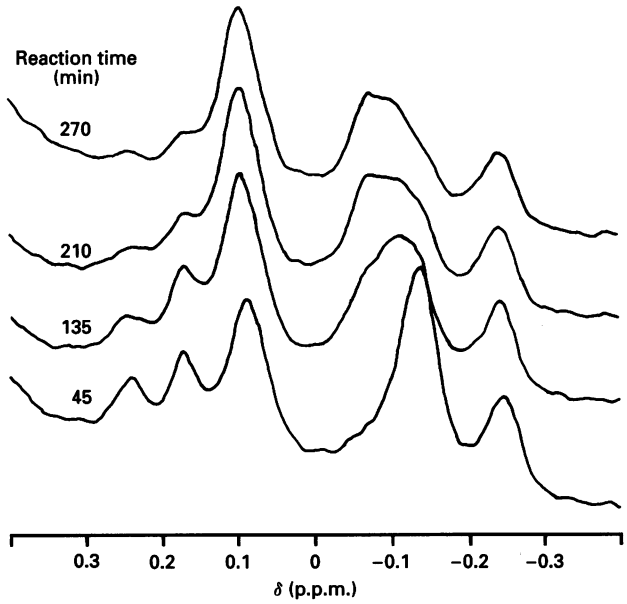


Figure 5 Progressive return of the modified enzyme to its initial configuration

The samples were those described in the legend of Figure 3.

$1.4 \times 10^{-4}\ s^{-1}$ respectively, in good agreement with that measured by monitoring the enzyme activity.

Interaction with other cephamycin-like compounds

The main difference between cephalothin and cefoxitin is the methoxy group on C-7. As the replacement of the acetyl group of cephalothin by the carbamyl group of cefoxitin is not expected to greatly affect the interaction with the enzyme, it was likely that the high stability of the acyl-enzyme formed with the latter was mainly due to the presence of the methoxy group. One should also note that the rate of acylation ($k_{cat.}/K_m = k_{+2}/K$) was also severely affected by the presence of the additional C-7 side chain. To confirm these deductions, two other compounds with a methoxy group on C-7 were also studied: moxalactam and FMCA. They were both poor substrates, as shown in Table 1, exhibiting very low acylation and deacylation rates. Both compounds transiently inactivated the enzyme, and the rate constants for re-activation were similar to $k_{cat.}$ in both cases, indicating that accumulation of acyl-enzyme also occurred. N.m.r. spectra recorded in the presence of moxalactam exhibited clear modifications, which were very similar to those induced by cefoxitin. However, the $k_{cat.}$ value for moxalactam indicated that the rate of deacylation with this compound was at least 7-fold faster than with cefoxitin, which made it unsuitable for multidimensional n.m.r. studies.

DISCUSSION

The results obtained in the present study underline the dramatic influence of a methoxy group on the α-face of a β-lactam on the interaction between the antibiotic and a class-A β-lactamase. Indeed, the only difference between temocillin and ticarcillin is the presence of the methoxy group on C-6 of the former. With the *B. licheniformis* enzyme, this resulted in a 2000000-fold decrease in the k_{+2}/K parameter, which characterizes the rate of acylation. Similarly, the k_{+3} value was decreased by more than

200000-fold, because the $k_{cat.}$ for ticarcillin only supplies a minimum k_{+3} value. Similarly, if one assumes that the slightly different C-3′ leaving groups of cephalothin and cefoxitin do not specifically influence their behaviour, the presence of the methoxy group on C-7 of the latter again decreased the k_{+2}/K and k_{+3} parameters by 100000-fold and more than 300000-fold respectively. These results were in excellent agreement with those reported by Matagne et al. (1993) for the same antibiotics and three other class-A β-lactamases, and by Fisher et al. (1980) and Faraci and Pratt (1986) with the TEM2 and *S. aureus* PC1 enzymes respectively.

The behaviour of all these class-A β-lactamases was in sharp contrast with that of class-C enzymes. Indeed, for five of these latter enzymes and cefoxitin, acylation was quite fast (k_{+2}/K = 0.04–2.5 $\mu M^{-1} \cdot s^{-1}$) and deacylation, although rather slow in absolute terms (0.01–0.3 s^{-1}), was always distinctly faster than for the class-A β-lactamases (Galleni et al., 1988).

With the *B. licheniformis* enzyme and cefoxitin, the only observable intermediate was the rearranged acyl-enzyme I. Indeed, within the limits of experimental error, the increase in A_{320}, which is characteristic of the appearance of the exomethylene group, appeared to be simultaneous with the opening of the β-lactam ring, as monitored by the decrease in A_{260}. This indicated that the elimination of the C-3′ leaving group was concomitant with the opening of the β-lactam ring or that the first, non-rearranged acyl-enzyme had an extremely short half-life. The results of Pratt and Faraci (1984) and Faraci and Pratt (1985) indicate that the second possibility probably prevails.

The n.m.r. studies highlighted the conformational changes in enzyme structure on formation of the acyl-enzyme. These were particularly striking in the high-field methyl-group area and could not be explained by magnetic anisotropy of the ligand itself. Nuclear Overhauser effect experiments (C. Damblon, unpublished work) gave further support to this interpretation.

Structural modification of other class-A β-lactamases [TEM1 (Citri et al., 1984), TEM2 and *S. aureus* PCI (Faraci and Pratt, 1986)] have also been assumed to explain the fact that acylation by cefoxitin increased the sensitivity of these enzymes to proteolytic agents. In the present paper, some modifications were indeed directly visualized by ^{1}H-n.m.r. Conversely, no delay was observed between deacylation and the recovery of enzymic activity, in contrast with the results of Citri et al. (1984), which were, however, obtained with another enzyme.

The present conclusions about the 7-methoxycephalosporins can probably be extended to the only 6-methoxypenicillin (temocillin) studied: this compound exhibited a very low $k_{cat.}$ and a rather high K_m value with the *B. licheniformis* β-lactamase. Similar poor recognition was recorded for three other class-A enzymes (Matagne et al., 1993) and, for a class-C β-lactamase, both acylation and deacylation being rather slow (D. Monnaie and J.-M. Frère, unpublished work).

From the c.d. spectra, it can be concluded that no major modification of the secondary-structure elements of the enzyme occurred on acylation by cefoxitin. Nevertheless, the protein crystals did not survive soaking in the antibiotic solution. Although the kinetic parameters appeared to be adequate, this result unfortunately rendered X-ray-crystallographic analysis of the acyl-enzyme impossible. One might hope to co-crystallize the enzyme with the antibiotic, but crystallization should then occur rapidly, before the concentration of cefoxitin decreases significantly.

In contrast, cefoxitin and the *B. licheniformis* β-lactamase represent an interesting substrate–enzyme system which is suitable for detailed ^{1}H-n.m.r. studies. These are at present underway, and the conformational changes observed on formation of the acyl-enzyme are receiving particular attention.

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