Hydrogen bonding and protein perturbation in β-lactam acyl-enzymes of *Streptococcus pneumoniae* penicillin-binding protein PBP2x

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A soluble form of *Streptococcus pneumoniae* PBP2x, a molecular target of penicillin and cephalosporin antibiotics, has been expressed and purified. IR difference spectra of PBP2x acylated with benzylpenicillin, cloxacillin, cephalothin and ceftriaxone have been measured. The difference spectra show two main features. The ester carbonyl vibration of the acyl-enzyme is ascribed to a small band between 1710 and 1720 cm^{-1}, whereas a much larger band at approx. 1640 cm^{-1} is ascribed to a perturbation in the structure of the enzyme, which occurs on acylation. The protein perturbation has been interpreted as occurring in β-sheet. The acyl-enzyme formed with benzylpenicillin shows the lowest ester carbonyl vibration frequency, which is interpreted to mean that the carbonyl oxygen is the most strongly hydrogen-bonded in the oxyanion hole of the antibiotics studied. The semi-synthetic penicillin cloxacillin is apparently less well organized in the active site and shows two partially overlapping ester carbonyl bands. The penicillin acyl-enzyme has been shown to deacylate more slowly than that formed with cloxacillin. This demonstrates that the natural benzylpenicillin forms a more optimized and better-bonded acyl-enzyme and that this in turn leads to the stabilization of the acyl-enzyme required for effective action in the inhibition of PBP2x. The energetics of hydrogen bonding in the several acyl-enzymes is discussed and comparison is made with carbonyl absorption frequencies of model ethyl esters in a range of organic solvents. A comparison of hydrolytic deacylation with hydroxaminolysis for both chymotryspin and PBP2x leads to the conclusion that deacylation is uncatalysed.

Key words: antibiotic, benzylpenicillin, infrared spectroscopy, transpeptidase.

INTRODUCTION

Microbial resistance to β-lactam antibiotics can arise from several sources. The best-known and most commonly encountered form arises from the expression of β-lactamases on the outside of bacteria [1]. The array of β-lactamases that have been selected is formidable, with the more recent members able to inactivate essentially all clinically used β-lactam compounds. Great efforts have been expended in determination of the structures of these enzymes, both as the apo forms and as acyl-enzyme complexes, formed with β-lactam antibiotics and inhibitors [2,3].

Not all clinically important bacteria produce β-lactamases, e.g. *Streptococcus pneumoniae*, and these species have developed resistance by a different route, as described below. Selection of resistant strains has also occurred through the use of β-lactamase-resistant antibiotics, such as the methicillin, which has selected for highly resistant strains of *Staphylococcus*.

In bacterial killing, β-lactam antibiotics act as analogues of the substrates of transpeptidase enzymes that catalyse the final event in the cross-linking of the bacterial cell wall and acylate the enzyme, forming stable inactive complexes [4,5]. Resistance has developed by the evolution of transpeptidases that are less susceptible to acylation by β-lactam antibiotics, while retaining a sufficient capacity to catalyse cell-wall cross-linking. This is a surprising ability, to avoid reaction with a good substrate analogue while retaining the ability to catalyse the natural reaction; this is a tightly focused discrimination. Acylation-resistant transpeptidases have evolved in the *Streptococci* and *Gonococci*, not only by point mutation but also by conjugation between closely related bacterial species with the 'importation' of segments of sequence from other species, leading to the creation of mosaic proteins that can be up to 1000 times less susceptible to acylation by β-lactams than the wild-type proteins [6].

Analysis of the crystallographic structures of native and resistant forms of the transpeptidases will form a major plank in the quest for an understanding of the mode by which resistance is acquired. IR spectroscopy can provide complementary information, often rapidly, concerning protein structure and changes therein, as well as on the nature and bonding in reaction intermediates. IR spectroscopic studies of chymotrypsin acyl-enzymes and substrate complexes of several other enzymes have demonstrated how such factors as the conformational distribution and hydrogen bonding of bound ligands can be elicited [7]. Hydrogen bonding of the ligand ester carbonyl to the oxyanion hole catalytic device of serine proteinases can be estimated from the frequency of the unique ester carbonyl vibration in acyl-enzymes, which decreases on hydrogen bonding, and isotope-editing can be used to assure assignments [7–10]. Fortunately, isotope editing is not required for most IR spectroscopic studies of acyl-enzymes formed between β-lactams and either β-lactamases or transpeptidases, since the ester frequency is well clear of strong protein amide absorbances.

Hydrogen bonding to the carbonyl oxygen atom of a carbonyl group lowers the IR stretching frequency of carbonyl groups by weakening the bond, whereas a low dielectric constant environment has the opposite effect of increasing the frequency. Separation of the influences of these two factors, although they

Abbreviations used: pM, pH meter reading in a ^2H_2O solution; IPTG, isopropyl β-D-thiogalactoside.

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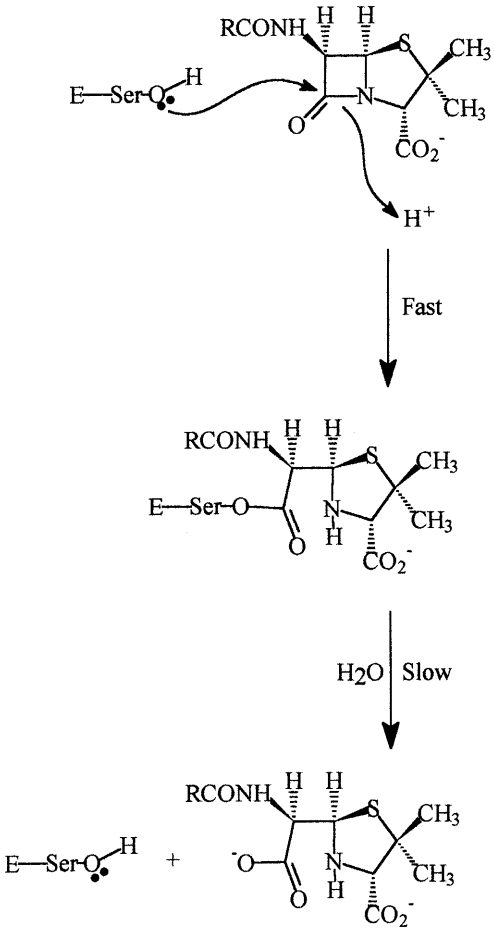

Scheme 1

Scheme 1 Schematic representation of the acylation and deacylation reactions of transpeptidases with penicillins

E, enzyme; Ser, serine.

have opposite effects, remains a challenge, but inspection of crystallographic structures and measurements made upon model esters in solvents of variable dielectric constant and hydrogen bonding ability can often help in interpretation [8]. The bandwidth of an IR spectroscopic feature reports upon the dispersion and/or mobility of the group responsible for the absorption and in many enzyme complexes a narrow bandwidth, compared with a suitable model compound in free solution, has been taken to represent a well-defined binding mode for a given ligand [11].

The amide groups of proteins absorb in the IR over a broad range from 1600 cm^{-1} to 1700 cm^{-1}, whereas the amide groups that comprise the various forms of secondary structure and absorb in this range have characteristic but overlapping absorption frequencies. The overall broad bandshape is frequently rather featureless but nonetheless many investigators have applied 'resolution enhancement' and band-fitting techniques in order to attempt to extract the underlying structural information [12].

In this paper we describe the application of the above techniques to the study of acyl-enzymes of the genetically modified, soluble form of *S. pneumoniae* PBP2x [13,14]. The wild-type enzyme has been modified by removal of the N-terminal membrane-anchoring domain, which confers solubility without compromising enzymic activity. The acylation and deacylation processes, which form the focus of this work, are shown in outline in Scheme 1. Two penicillins and two cephalosporin antibiotics have been used to form acyl-enzyme complexes. Structures of the compounds employed in this study are shown as Figure 1. A comparison of hydrogen bonding and ligand mobility is made between 'natural' and semi-synthetic penicillins as well as cephalosporins, and the implications of hydrogen bonding are discussed.

MATERIALS AND METHODS

Benzylpenicillin, ampicillin, cloxacillin, ceftriaxone, cephalothin, ^2H_2O and Hepes buffer were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.) and used as received. Sodium ethoxide (3.4 M) in ethanol was supplied by Fluka (Gillingham, Dorset, U.K.).

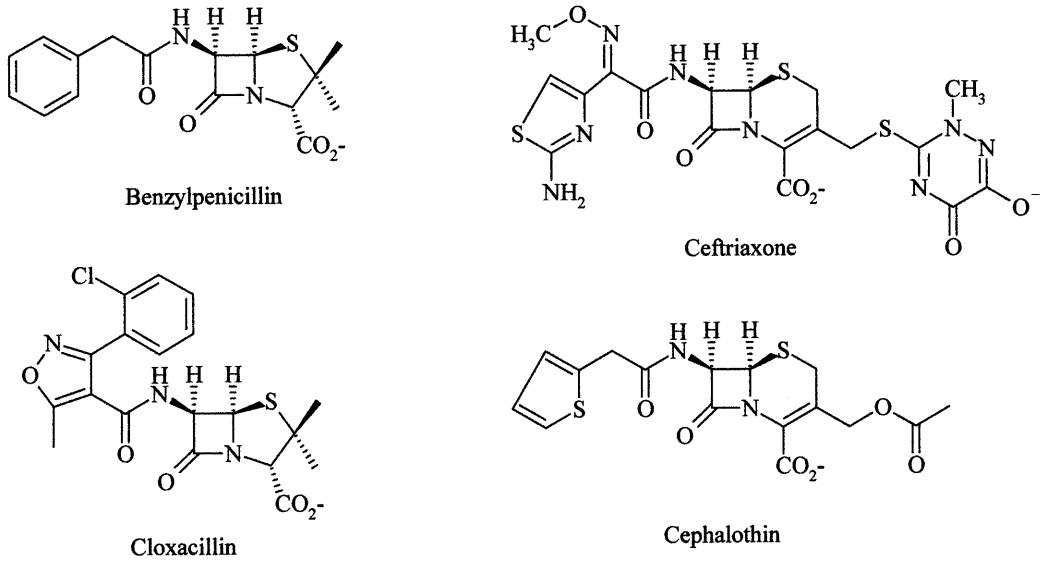

Figure 1 The structures of the penicillins and cephalosporins used for IR spectroscopy

Expression and purification of recombinant PBP2x

Construction of the *Escherichia coli* expression plasmid pMC56

The expression plasmid pDS56/RBSII [15] contains as selection marker the β-lactamase gene of plasmid pBR322. Since this gene could interfere with the analysis of the overproduced penicillin-binding protein, the β-lactamase gene was deleted from the expression plasmid. Two DNA fragments were isolated from plasmid pDS56/RBSII; (i) a 336 bp long *Aat*II–*Nhe*I fragment on to which a *Bgl*II linker was ligated at the *Nhe*I site, and (ii) a 1939 bp long *Nhe*I–*Sau*3A fragment on to which an *Aat*II linker was ligated at the *Nhe*I site. Both fragments were ligated to each other leading to plasmid pMC56, which has the chloramphenicol acetyltransferase gene in opposite orientation compared with pDS56/RBSII and which does not contain the β-lactamase gene. All cloning, plasmid constructions and PCR reactions were performed using methods described previously [16,17].

Construction of the *E. coli* expression plasmid pMC56,PBP2x

The gene encoding the soluble form of PBP2x from *S. pneumoniae*, located on plasmid pCG31 [18], was obtained from Dr. R. Hakenbeck (Max-Planck Institut für molekulare Genetik, Berlin, Germany). The gene codes for a protein of 750 amino acids and contains a deletion of amino acids 19–48, representing the hydrophobic N-terminal domain of PBP2x. Using PCR technology, the PBP2x gene was placed under the control of the isopropyl β-D-thiogalactoside (IPTG)-inducible promoter present on pMC56 leading to plasmid pMC56,PBP2x (Δ19–48). All cloning, plasmid constructions and PCR reactions were performed using methods described previously [16,17].

Overproduction of the PBP2x (Δ19–48) construct in *E. coli* K12

The protein was expressed in *E. coli* strains M15 or W3110 (American Type Culture Collection 27325). The lac repressor plasmid pREP4 [15] contains the DNA replication region (ori) of plasmid pACYC184, which is compatible with the pBR322 replication region (ORI) present in the pMC56 expression vectors. Cultures of *E. coli* cells harbouring a combination of the repressor plasmid pREP4 and the expression plasmid pMC56,PBP2x (Δ19–48) were grown in Luria–Bertani medium [17] containing 5 μg/ml chloramphenicol and 25 μg/ml kanamycin at 37 °C. At a D_{600} of 0.7–0.8, IPTG was added to a final concentration of 1 mM and the cultures were further incubated for 3 h. The cells were then harvested and the cell paste frozen for storage at −80 °C.

Purification of PBP2x

Approximately 50 g of frozen cell paste was broken into small pieces and thawed in 200 ml of cold (0–10 °C) 20 mM Tris/HCl, adjusted to pH 7.8. The following additions (final concentration) were made: bovine pancreatic DNAase (1 μg/ml)/chymostatin (1 μg/ml)/antipain (5 μg/ml)/pepstatin (0.1 μg/ml)/leupeptin (1 μg/ml)/aprotinin (0.1 unit/ml)/$MgSO_4$ (10 mM)/ϵ-aminohexanoic acid (5 mM). The suspension was passed through a pre-cooled French press cell at about 2000 MPa and then centrifuged at 100000 ***g*** for 90 min at 4 °C. The supernatant was reserved for column chromatography, as described below, while the pellet was resuspended in 200 ml of 20 mM Tris/10 mM NaEDTA, adjusted to pH 9.0 with H_2SO_4. The suspension was stirred at 4 °C and then centrifuged at 100000 ***g*** for 90 min at 4 °C. This extraction was repeated twice, the supernatants being reserved for column chromatography. The supernatants having the greatest amount of PBP2x were identified by labelling the supernatants with fluorescent penicillin analogues [19,20] and analysis using SDS/PAGE. These fractions, usually the first and second extractions with 20 mM Tris/10 mM NaEDTA, pH 9.0, were taken for chromatography. The extracts were applied to a column (400 ml bed volume) of DEAE-Sepharose CL-6B equilibrated in 20 mM Bis-Tris/propane/1 mM NaEDTA, adjusted to pH 8.0 with H_2SO_4. The column was washed with 5 volumes of equilibration buffer and then the proteins were eluted with a three-step NaCl gradient of 0–0.2 M (400 ml), 0.2–0.7 M (300 ml) and 0.7–1.0 M (300 ml) in equilibration buffer at a flow rate of 1 ml/min. The fractions containing active protein were identified by fluorescence-labelling and gel electrophoresis as before. These fractions were pooled, concentrated and dialysed against 0.1 M sodium phosphate buffer, pH 7.0, for application to a Pharmacia Superdex 200 Hiload 26/60 column, equilibrated in 0.1 M sodium phosphate buffer, pH 7.0. The protein was eluted with the same buffer at a flow rate of 1 ml/min. The fractions containing pure PBP2x were identified by SDS/PAGE. The enzyme solution was stored in a 50% glycerol solution at −20 °C prior to use.

Preparation of PBP2x for IR experiments

To remove glycerol prior to freeze–drying, 25 ml of PBP2x in 50% glycerol/100 mM sodium phosphate buffer, pH 7.0, was diluted with 25 ml of water and reduced to 3–5 ml by pressure dialysis, using a 10 kDa cut-off filter. Phosphate buffer (pH 7.0, 10 mM, 50 ml) was added and the solution was concentrated to 3–5 ml by pressure dialysis. The dilution and re-concentration was repeated again prior to freeze–drying of the protein. The enzyme was dissolved in 100 mM phosphate buffer, pM 7.0 (where pM is the pH meter reading in a ^2H_2O solution), made up in ^2H_2O to give a concentration of 0.5–1.5 mM and incubated at 4 °C for 48 h prior to experiments to ensure extensive hydrogen–deuterium exchange of the protein.

Measurement of the proportion of PBP2x that is capable of acylation

Titration with nitrocefin was routinely used to assay the concentration of active enzyme to be used in IR experiments. Measurements were made using 100 mM phosphate buffer, pH 7.0, and the instantaneous change in absorbance at 482 nm upon mixing enzyme and nitrocefin was determined. The enzyme concentration in the assays was 2–3 μM, and nitrocefin was 5 μM. The value of $\Delta\epsilon_{482}$ used to determine the active enzyme concentration was 17400 $M^{-1}\cdot cm^{-1}$. Measurements made on acyl-enzymes showed that > 90% of the enzyme was acylated in all experiments.

Ethanolysis of antibiotics

Antibiotic (0.1 g) was dissolved in 10 ml of ethanol and 0.1 molar equiv. of sodium ethoxide in ethanol was added. As shown by TLC and IR analysis the reactions were complete within 5 min, at which time 0.1 equiv. of glacial acetic acid was added to neutralize the base. Aliquots (1 ml) of the ethyl ester solutions were evaporated under a stream of dry nitrogen and redissolved in 1 ml of a range of solvents (see below, Figure 6) for the determination of the effect of solvent dielectric constant upon the frequency of the ester carbonyl absorbance.

IR spectroscopic experiments

To 95 μl of 0.2–1.5 mM enzyme solution was added a volume of 20 mM stock antibiotic solution in ^2H_2O, which represents a 0.9–1.1-fold excess concentration of antibiotic over the enzyme.

Thus, if the enzyme solution was exactly 1 mM, as determined by nitrocefin titration, 5 μl of 20 mM antibiotic solution was added.

For the sample spectrum, 100 μl of acylated enzyme was injected into an *in situ* IR cuvette using a Hamilton HPLC syringe. The cuvette has CaF_2 windows and a pathlength of 50 μm. This arrangement allows filling, emptying and washing of the cuvette without having to break the dry-air purge of the IR spectrometer. For the reference spectrum a similar volume of unacylated enzyme was injected into the IR cuvette. In some experiments a spectrum of 100 mM phosphate buffer, pM 7.0, in ^2H_2O was also collected for use as a reference in the determination of independent spectra of the free enzyme and acyl-enzymes.

Spectra were obtained using a Bruker IFS 66 spectrometer and 512 scans at 2 cm^{-1} resolution were collected for each of the sample and reference spectra. A liquid nitrogen-cooled mercury-cadmium-telluride detector was used.

Enzyme and acyl-enzyme spectra were obtained from a ratio of sample or reference spectra divided by the ^2H_2O background spectrum, and difference spectra by subtracting the reference from the sample. Alternatively, and more usually, difference spectra were constructed from a ratio of the sample spectrum divided directly by the reference (free-enzyme) spectrum. When subtraction was used the factor was always unity, i.e. there was no attempt to 'adjust' the spectra by means of variable subtraction. Unless otherwise stated, no smoothing or base-line correction was applied to the spectra.

RESULTS AND DISCUSSION

The spectra of uncomplexed PBP2x and the acyl-enzyme formed upon reaction with benzylpenicillin are shown in Figure 2. It is notable that differences are directly observable in the raw spectra and comprise features at approx. 1712 and 1640 cm^{-1}. The feature at 1712 cm^{-1} is in the carbonyl stretching region characteristic of an ester or protonated carboxyl group, whereas the distinct feature at approx. 1640 cm^{-1} probably represents a change in the protein amide spectrum as a result of interaction between the ligand and the protein. It is possible to define the frequency maxima accurately as 1712 and 1640 cm^{-1} in the difference spectrum (Figure 3). The relative magnitudes of the bands can also be compared. It is immediately apparent that the feature at 1640 cm^{-1} is much larger than the one at 1712 cm^{-1}. This indicates that it arises predominantly from perturbation of a number of protein amide carbonyl groups.

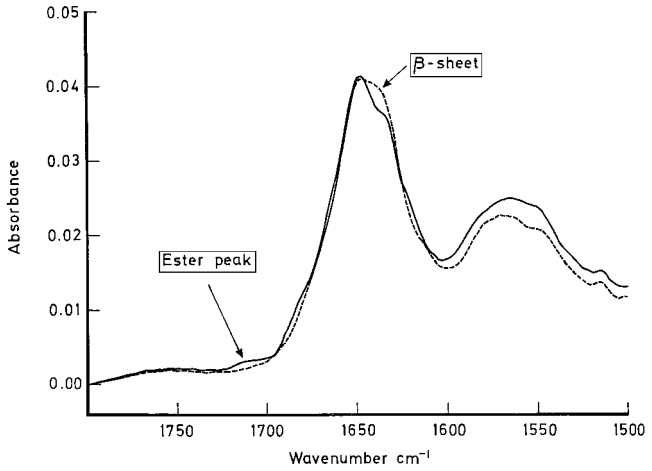

Figure 2 IR spectra of PBP2x and in complex with benzylpenicillin

Dashed line, free enzyme; solid line, acyl-enzyme. The enzyme concentration was 0.22 mM at pM 7.0 in 100 mM phosphate buffer in each spectrum. For this experiment the 20 mM stock benzylpenicillin was diluted to 2.5 mM in ^2H_2O prior to addition of 10 μl to 90 μl of enzyme solution. ^2H_2O (10 μl) was added to 90 μl of enzyme solution for measurement of the free-enzyme spectrum. For each spectrum 512 scans were accumulated at 2 cm^{-1} resolution. The reference spectrum was 100 mM phosphate buffer in ^2H_2O in each case.

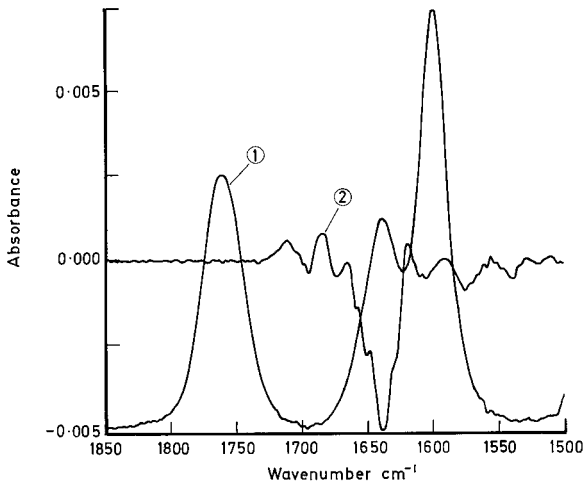

Figure 3 Superimposed spectra of benzylpenicillin (1) and the unprocessed PBP2x–acyl-enzyme difference spectrum (2), generated by subtraction of the two traces shown in Figure 2

The acyl-enzyme ester peak at 1712 cm^{-1} and the large protein perturbation peak (relative to the ester band) at 1640 cm^{-1} are seen clearly. The benzylpenicillin spectrum, determined in the same buffer as the enzyme spectra at a concentration of 20 mM, is not scaled to the difference spectrum and is provided as an indication of peak positions only. The band at 1640 cm^{-1} in the benzylpenicillin spectrum represents the side-chain amide group, whereas that at 1600 cm^{-1} is assigned to the carboxylate group.

The IR spectrum of benzylpenicillin in ^2H_2O is shown in Figure 3. The β-lactam ring carbonyl absorbs at a higher frequency (1753 cm^{-1}) than both ester and protonated carboxyl groups and also at a much higher frequency compared with the protein amide groups (1610–1700 cm^{-1}). All β-lactam carbonyl groups absorb with very similar frequencies, which are well clear of protein amide bands (see below). The high-frequency absorbance of the lactam carbonyl may be ascribed to the lack of resonance stabilization in the lactam ring. This also has the consequence that the C–N bond is weaker than in a normal amide. The side-chain amide group absorbs at 1641 cm^{-1} in the spectrally dense region of the protein amide carbonyl groups. The strong band at 1603 cm^{-1} is assigned to the carboxylate group.

Ligand-induced perturbation of PBP2x structure

It has been established from studies of peptides that the different types of secondary structure have characteristic IR absorption frequencies. Thus, β-sheet has strong absorbance at 1630–1640 cm^{-1}, whereas α-helix absorbs at approx. 1655 cm^{-1} (although there can be exceptions). In principle, parallel and antiparallel sheets can be distinguished and also the presence of turns can be identified [12]. The amide absorption of globular proteins commonly forms a broad, rather featureless, envelope. A variety of computational methods have been used to identify and quantify underlying bands that are characteristic of specific types of secondary structure [12]. These methods have been calibrated against known X-ray structures.

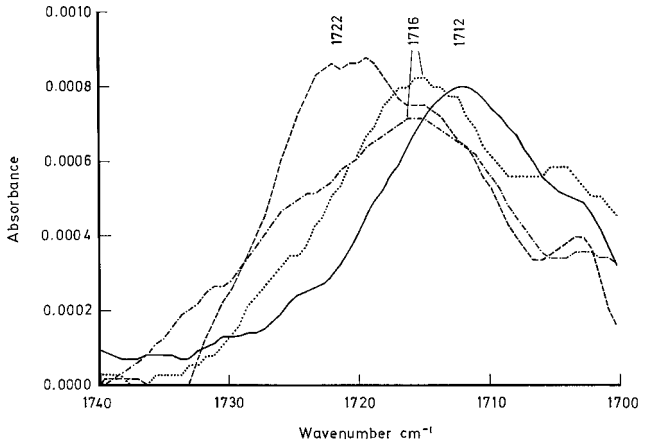

Figure 4 Unprocessed ester carbonyl bands of PBP2x acyl-enzymes

Dashed line, cephalothin; dot–dash line, cloxacillin; dotted line, ceftriaxone; solid line, benzylpenicillin. The enzyme concentration was 0.22 mM and the enzyme was ≈ 90% acylated in each case.

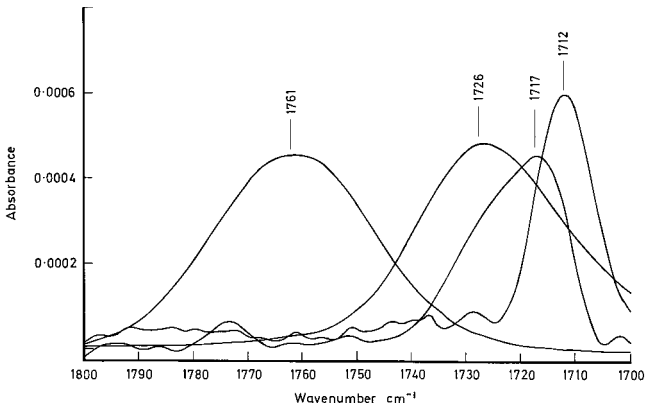

Figure 5 Base-line-corrected and smoothed spectra of (left to right): benzylpenicillin (1761 cm^{-1}), ethyl penicilloate (1726 cm^{-1}), PBP2x-cloxacillin acyl-enzyme (1716 cm^{-1}) and PBP2x-benzylpenicillin acyl-enzyme (1712 cm^{-1})

The intensities of the benzylpenicillin and ethyl ester spectra have been scaled to that of the cloxacillin acyl-enzyme, which was measured at 0.22 mM, the same concentration as the benzylpenicillin acyl-enzyme. Experimental details as given in Figure 2 and the Materials and methods section.

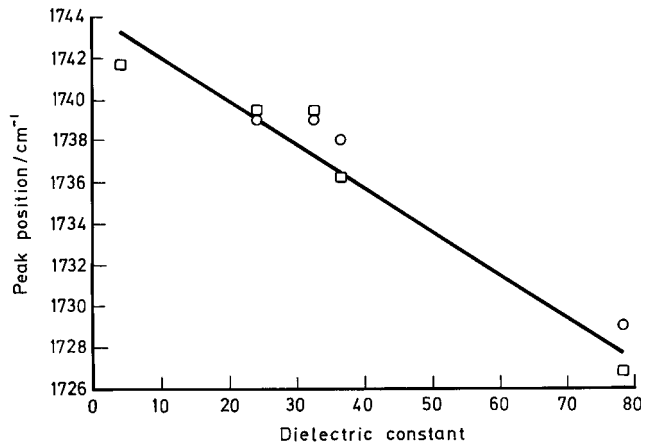

Figure 6 Plot of the ester carbonyl frequency maximum of ethyl esters of benzylpenicillin (○) and ampicillin (□) against the solvent dielectric constant

The ethyl esters and the solutions in various solvents were prepared as described in the Materials and methods section. Note that all the frequencies are well above those observed in acyl-enzymes. Solvents were: diethyl ether ($D = 4$); methanol ($D = 33$); acetonitrile ($D = 37$); ethanol ($D = 24$) and ^2H_2O ($D = 78$). In ethanol and methanol the esters show two carbonyl frequencies of which the lower one is plotted here.

A change in structure/mobility is relatively easily identified and quantified when it is largely restricted to the β-structure, as seen for PBP2x in Figure 3. The difference spectrum shows that the absorbance in the uncomplexed enzyme is stronger than in the acyl-enzyme. This may tentatively be interpreted to indicate that the β-structure is disrupted in the acyl-enzyme relative to the free enzyme. The disruption is predicted to occur immediately adjacent to the active site, bordering one edge of the cleft [14]. The regular hydrogen bonding of the sheet may be disrupted by a 'rolling over' of the edge of the sheet, which will result in a loss of organized hydrogen bonding and a consequent loss of IR intensity. It is interesting to question why the 'lost' absorbance does not show up elsewhere in the difference spectrum as a positive feature, indicating the formation of a new type of structure. We assume that a distortion of β-sheet, rather than transformation to another type of structure, e.g. random coil, causes a loss in absorbance due to disruption of hydrogen bonding rather than movement to a new frequency. Alternatively, the 'new' absorbance, if such exists, may be distributed among a wide range of frequencies and so be sufficiently broadened to be invisible. From the band area the perturbation is estimated to affect 5–10% of the β-sheet in the protein.

The ester carbonyl band

The band at 1712 cm^{-1} in Figures 3, 4 and 5 is assigned to the carbonyl of the stable ester that is formed upon acylation with benzylpenicillin. It remains a remote possibility that the band could arise from a perturbation of the pK_a of a carboxyl group with an abnormally high pK_a, such that the fraction of protonated form is increased in the acylated enzyme; i.e. the pK_a is raised in the acyl-enzyme relative to the free enzyme. Such an effect has been observed in IR studies of substrate complexes of the enzyme aldolase at pD 7.4, with the putative perturbed carboxyl group showing maximal absorbance at 1748 cm^{-1} [21]. A similar effect is seen at pD 4.0 in chymotrypsin acyl-enzymes, where the perturbation has a frequency maximum of 1737 cm^{-1} in hydrocinnamoyl chymotrypsin [9]. Presumably, in each case, one or more carboxyl groups experience a more hydrophobic environment in the enzyme complex compared with that in the free enzyme. This is an extremely unlikely explanation of the carbonyl band we assign to the acyl-enzyme ester carbonyl groups because the frequency of the putative ester carbonyl band is much lower than the frequencies that arise from the perturbations described above (see Figures 4 and 5). The observed ester frequency is in the region where carboxylic acids absorb in aqueous solution. Acetic acid absorbs at 1710 cm^{-1} in ^2H_2O and at 1760 cm^{-1} in carbon tetrachloride. It is not easy to conceive of an enzymic carboxyl group with a p$K_a \geqslant 7.0$, so that it would be partially protonated at the pH of our experiments, in a hydrophilic environment.

A series of ester carbonyl absorption bands from difference spectra of several acyl-enzymes in unprocessed form is shown in

Figure 4. It is clear that the intensity of absorption of each is similar and indeed the ester absorbance is similar to the β-lactam carbonyl absorption of the antibiotics. The ester carbonyl frequency of the benzylpenicillin acyl-enzyme is clearly the lowest, which is taken to indicate the strongest hydrogen bonding in the oxyanion hole (see below). The band envelope for cephalothin possesses at least two elements. The largest peak at 1722 cm^{-1} arises from the ester group of the 3′-acetyl group that comprises part of the structure of cephalothin, whereas the smaller component at approx. 1716 cm^{-1} is presumed to represent the acyl-enzyme carbonyl. Thus, the ester frequencies and hence hydrogen bonding for the semi-synthetic cloxacillin and the two cephalosporins are very similar. Several of the spectra appear to have a feature between 1700 and 1704 cm^{-1}. Repeating experiments indicated that this is variable, so it can only be suggested very tentatively that these might represent less populated but more strongly hydrogen-bonded conformers.

Smoothed and base-line-corrected spectra derived from those presented in Figures 3 and 4 are shown in Figure 5. It is apparent that the benzylpenicillin acyl-enzyme band ($\nu = 1712$ cm^{-1}) is narrower than the others; indeed, the contrast with the model ester and β-lactam carbonyl bands is dramatic. A narrow symmetrical band in IR spectra implies a well-ordered structure in a single conformation, so the ring-opened ester form of this antibiotic is very well ordered in the enzyme active site, in part as a result of good hydrogen bonding in the oxyanion hole. Thus, both the low frequency and the narrowness of the band support the concept, well supported by kinetic studies, that benzylpenicillin acylates the enzyme rapidly. By contrast, the cloxacillin band is asymmetric and, although the components are overlapped, shows evidence of more than one conformation. The more intense of these bands coincides with the cephalosporin frequencies as seen in Figure 4, while the less intense one appears to coincide with the frequency shown by the penicillin model esters ($\nu = 1727$ cm^{-1}) in ^2H_2O. There appears to be a less populated conformer in which the carbonyl group experiences a hydrogen bonding/dielectric constant environment characteristic of solvent. In this conformer, the ester carbonyl is exposed to solvent, i.e. projecting out of the active site away from the oxyanion hole, or it is more weakly hydrogen-bonded to the oxyanion hole.

It is interesting that the ester carbonyl of the benzylpenicillin acyl-enzyme is the most strongly hydrogen-bonded. Indeed the hydrogen bonding is significantly stronger than in aqueous medium, since the frequency is lower. This may be simply a 'leftover' consequence of the transition-state stabilization provided by the oxyanion hole during acylation or may serve to stabilize the acyl-enzyme and so enhance bacterial killing. The benzylpenicillin acyl-enzyme is more stable to deacylation (0.029 h^{-1}) than is the semi-synthetic cloxacillin (0.17 h^{-1}), and benzylpenicillin acylates the enzyme with a slightly higher second-order rate constant. The interactions with cephalosporins are more complex as they are known to undergo elimination of the 3′-side chain after acyl-enzyme formation [22]. Cefotaxime acylates the enzyme about twice as fast as the penicillins but deacylates at one-fiftieth of the rate [13]. However, the cephalosporins in our study do not apparently hydrogen bond as well as benzylpenicillin.

The strength of the hydrogen bonds

An approximate estimate of hydrogen-bonding strength to a carbonyl oxygen atom can be obtained from an estimate of the loss of strength in the carbonyl group, and this can be estimated from the IR spectral shifts of model compounds in a range of solvents, as shown in Figure 6 and in acyl-enzymes [8]. In all acyl-enzymes considered here, the hydrogen bonding is stronger than that in aqueous solution, since the frequencies are lower, so the hydrogen-bonding strength is estimated by relation to the model ester in an apolar solvent (all penicillin-derived model esters have the same ester carbonyl frequencies in a range of solvents; see Figure 6). Penicillin esters can epimerize at C5 and C6 in strongly basic conditions. A small amount of epimerization may occur at C5 during preparation of the model esters used here but this will not affect the IR absorbance frequency of the ester carbonyl. Epimerization at C6 is too slow to have occurred in our procedures [23–25].

A simple relationship between bond strength and the frequency of vibration of a diatomic oscillator, to which the carbonyl stretch vibration approximates quite well since it is a local mode, can be derived from the Morse potential [26]. This states that $D_e = \omega_e/4x_e$, where D_e is the dissociation energy of the bond (plus the zero point energy), ω_e is the frequency of the vibration and x_e is the anharmonicity constant. For a shift in frequency we can write $\Delta D_e = \Delta\omega_e/4x_e$ and, combining this equation with the original one by division, we can eliminate $4x_e$, giving: $\Delta D_e = (\Delta\omega_e/\omega_e)\cdot D_e$.

The shift in frequency between diethyl ether (1742 cm^{-1}) and ^2H_2O (1727 cm^{-1}) is 15 cm^{-1} and the carbonyl bond strength is approx. 730 kJ/mol, so assuming two hydrogen bonds from water (^2H_2O) to each carbonyl oxygen atom, each of the bonds is worth approx. $(0.5 \times 730 \times 15)/1747 = 3.2$ kJ/mol. The shift from ^2H_2O to the benzylpenicillin acyl-enzyme is also 15 cm^{-1}, which means that each hydrogen bond is approx. 6.4 kJ/mol in the acyl-enzyme. The total hydrogen bonding is thus a modest 12.8 kJ/mol. Interestingly, this value is similar to that estimated (14 kJ/mol) for a 30 cm^{-1} shift from a spectroscopic-kinetic correlation between the carbonyl absorption frequencies and deacylation rate constants for the acyl groups of a series of serine proteinase acyl-enzymes constructed from Raman spectroscopic experiments by Carey and Tonge [27]. The hydrogen bonding seen here, if expressed in kinetic terms as a potential stabilizing or reactive influence, is worth a factor of some 170-fold. If used to stabilize the acyl-enzyme it would decrease the half-time for deacylation from 9 min to the observed 24 h.

That the natural benzylpenicillin hydrogen-bonds most strongly seems to be another example of the principle that 'Nature knows best', in that the natural compound seems to interact with the enzyme most precisely. Hydrogen bonding in the oxyanion hole is usually associated with the induction of electrophilicity at the carbonyl carbon, as required, for example, in facile acylation. In the case of the PBP2x acyl-enzyme with benzylpenicillin it appears that the hydrogen bonding is used to stabilize the acyl-enzyme and so enhance antibiotic potency rather than to promote deacylation. We expect quite the opposite in the case of β-lactamases, where such hydrogen bonding is predicted, by analogy with the serine proteinases, to accelerate deacylation.

Deacylation of benzylpenicillin acyl-enzyme

Whereas the hydrolytic deacylation reaction is slow and has been reported [28] to proceed with cleavage of the C5–C6 bond, hydroxaminolysis is much faster. At pM 7.0 the first-order constant for hydrolytic deacylation (in ^2H_2O), measured as the disappearance of the 1712 cm^{-1} acyl-enzyme band (results not shown) is 4.0 $(\pm 0.3) \times 10^{-6}$ s^{-1}, whereas for hydroxaminolysis the second-order constant is 1.82 $(\pm 0.08) \times 10^{-2}$ $M^{-1}\cdot s^{-1}$. The ratio of hydroxaminolysis/hydrolysis (H_2O) is thus approx. 4500 M^{-1}, which compares with the ratio of 500 M^{-1} for furoyl

chymotrypsin [29]. Furoyl chymotrypsin is a non-specific stable acyl-enzyme with a rate constant for deacylation that is approx. 10^5-fold slower than for the highly specific acetyl-L-tyrosyl group [30]. The hydroxaminolysis/hydrolysis ratio for acetyl-L-tyrosyl chymotrypsin is a mere 1.3 M^{-1}, which shows that the ratio is very dependent upon the kinetic efficiency of the catalytic process [30]. Where catalysis of the hydrolytic process is highly specific, the large difference in inherent nucleophilicity (see below) between hydroxylamine ($pK_a = 6.2$) and water ($pK_{a1} = -1.2$) disappears, owing to the nucleophilic enhancement provided by the enzyme which transforms water towards being hydroxide ion-like. The ratio for *p*-nitrophenyl acetate of 3.0×10^6 M^{-1} demonstrates the large inherent difference in nucleophilicity between hydroxylamine and water, ascribed to the α-effect, which is entirely overcome by the enzyme [31]. Indeed, the second-order rate constants for hydroxaminolysis and hydrolysis by hydroxide ion of *p*-nitrophenyl acetate differ by less than a factor of 10 despite the 10^9-fold difference in basicity [pK_{a2} (H_2O) = 15.2]. The values presented above show clearly that PBP2x is very deficient in catalysis of deacylation, being much less effective than chymotrypsin towards a very poor substrate.

PBP2x lacks the residues (e.g. Glu-166) present in β-lactamases that are proposed to form the catalytic system for deacylation [14], so it is the intrinsic chemical reactivity of hydroxylamine that drives the reaction without catalysis. The attack of hydroxylamine is perhaps analogous to the natural transpeptidase activity of the enzyme. A chemically reactive partially unprotonated α-amino group is the incoming nucleophile, so a catalytic group will not necessarily be required to drive the reaction.

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