Identification of the site of covalent attachment of nafcillin, a reversible suicide inhibitor of β -lactamase

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Nafcillin was shown to reversibly inhibit β -lactamase from *Staphylococcus aureus* PC1 with characteristics indicative of a type A inhibitor [Citri, Samuni & Zyk (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1048–1052]. At nafcillin concentrations above 80 mM, complete inactivation occurred within 200 s. Upon removal of the excess nafcillin the inhibited enzyme was re-activated completely, with a rate constant of $2.0 \times 10^{-3} \text{ s}^{-1}$ (25 °C). The inhibited enzyme was shown to be in the form of a covalent acyl-enzyme intermediate. Digestion by pepsin and trypsin yielded a single nafcillin-labelled peptide fragment which was isolated, sequenced and shown to be: Ala-Tyr-Ala-Ser-Thr-Ser-Lys. This sequence corresponds to the region surrounding the active-site serine residue, Ser-70, indicating that the inhibitor is covalently attached to the same residue as productive substrates.

INTRODUCTION

β -Lactamase from *Staphylococcus aureus* PC1 is a class A β -lactamase whose major function is to hydrolyse the β -lactam bond in antibiotics such as penicillins and cephalosporins and is a major source of resistance to β -lactam antibiotics. This makes the study of inactivation of β -lactamases a clinically important issue, and may enable a rational approach to the design of more effective antibiotics. Certain semisynthetic penicillins, such as methicillin, have long been recognized as having inhibitory properties, owing to the induction of conformational change (Kiener & Waley, 1977; Pain & Virden, 1979; Kiener *et al.*, 1980). This type of substrate, which causes the enzyme to undergo a form of reversible suicide inhibition, has been called a 'type A substrate' by Citri *et al.* (1976). The presence of this type of inhibition is manifested by a time-dependent decrease in the enzyme activity, leading to a low level of steady-state catalytic activity due to residual active enzyme, followed by a return to full catalytic activity upon exhaustion of the substrate.

Previous investigations of class A β -lactamases with good substrates (Cartwright & Fink, 1982; Anderson & Pratt, 1983; Bicknell & Waley, 1985; Pratt *et al.*, 1988), and inhibitors (Knott-Hunziker *et al.*, 1979; Cohen & Pratt, 1980; Cartwright & Coulson, 1980; Fisher *et al.*, 1980; Clarke *et al.*, 1983), indicate that the catalytic reaction involves the intermediacy of an acyl-enzyme intermediate. Various studies have implicated Ser-70 as the nucleophile responsible for the formation of the acyl-enzyme (Fisher *et al.*, 1981; Dalbadie-McFarland *et al.*, 1982; Sigal *et al.*, 1982; Cartwright *et al.*, 1989; Virden *et al.*, 1990). However, with type A substrates (Citri *et al.*, 1976), the identity of the attachment site is still unknown (Virden *et al.*, 1975, 1978; Kiener *et al.*, 1980; Hardy & Kirsch, 1989). It is possible that type A substrates may form a covalent linkage at some site other than the essential serine residue, Ser-70, since Virden *et al.* (1978) reported multiple sites of attachment of the type A substrate quinacillin to staphylococcal β -lactamase.

The goals of the present study were: (1) to study the reaction between nafcillin and staphylococcal β -lactamase and to establish whether nafcillin is a normal or type A substrate with this enzyme; (2) to trap the putative acyl-enzyme intermediate; and (3) to identify the site of covalent linkage. Nafcillin was chosen

as a potential type A substrate because of the chromophoric C-6 side chain (a naphthalene derivative), which has significant absorbance in the visible region of the spectrum and can thus be used as an intrinsic label to locate the site of covalent attachment of the substrate to the enzyme.

MATERIALS AND METHODS

Materials

β -Lactamase from *Staphylococcus aureus* PC1 was generously given by Dr. Richard Virden, University of Newcastle upon Tyne. The preparation was homogeneous by SDS/PAGE. Nafcillin was purchased from Sigma, as the sodium salt. Trifluoroacetic acid (TFA), h.p.l.c. grade, was purchased from Pierce. Potassium phosphate was used in the preparation of all buffer solutions.

Methods

Enzymic hydrolysis of nafcillin. Spectra (260–345 nm) of nafcillin (0.4 mM) and its hydrolysed product [from the addition of β -lactamase (0.68 μM)] were recorded on a Perkin–Elmer model 320 spectrophotometer at pH 7.0 and at 20 °C. The enzymic hydrolysis of nafcillin was monitored at 325 nm. The decrease in the rate of hydrolysis of nafcillin due to the inhibition of the enzyme could be monitored directly by spectrophotometry. Cuvettes of 0.02 mm path length were used because of the high background absorbance. Typical conditions for the inactivation experiment were 7 μM - β -lactamase with 84 mM-nafcillin in 0.2 M-potassium phosphate buffer, pH 7.0, at 20 °C.

Effect of inhibitor concentration on amount of inactivation. The experimental conditions involved incubating 5 μM - β -lactamase with various concentrations of nafcillin at pH 7.0 (0.2 M-potassium phosphate buffer; 20 °C) for 4 min. Owing to high background absorbance, a portion of the incubation mixture was removed and diluted 100-fold. The enzymic activity was checked by assay against 2.2 mM-benzylpenicillin (a good substrate) at 240 nm.

Time course of inactivation. The kinetics of inactivation of the enzyme by nafcillin were determined as follows. β -Lactamase (7 μM) was incubated with 100 mM-nafcillin at 0.2 M-potassium

Abbreviation used: TFA, trifluoroacetic acid.

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phosphate buffer, pH 7.0 at 20 °C. A 100-fold-diluted portion of the incubation mixture was assayed with 2.2 mM-benzylpenicillin at 240 nm.

Kinetics of re-activation. The inactivated enzyme was prepared by incubating 8 μ M- β -lactamase with 100 mM-nafcillin at pH 7.0 in 0.2 M-potassium phosphate buffer at 20 °C for 4 min, which led to complete inactivation. The re-activation assay was conducted by adding a 100-fold-diluted portion containing the inactivated enzyme to a solution of benzylpenicillin (2.2 mM). The re-activation curve was recorded on a Perkin-Elmer 320 spectrometer at 240 nm and the data were analysed by the method of Koerber & Fink (1987).

Trapping of the acyl-enzyme intermediate. Since nafcillin was shown to be a reversible inhibitor of the staphylococcal β -lactamase, trapping of the enzyme-substrate intermediate was done at 20 °C, where the rate of re-activation was reasonably slow. The conditions under which the putative penicilloyl-acyl-enzyme was trapped were 10 μ M- β -lactamase and 100 mM-nafcillin in 1 ml of 0.2 M-potassium phosphate buffer, pH 7.0. The temperature of the incubation was 20 °C and the reaction was allowed to proceed for 3½ min. A portion (50 μ l) of 10% (w/v) trifluoroacetic acid (TFA) was then added to the incubation mixture. This was to lower the pH of the incubation to pH 5 in order to stabilize the inhibited enzyme. A mini-gel column (DEAE-Sephadex G-25; 1.3 cm $\times$ 5.0 cm) was equilibrated with 0.2 M-acetate buffer, pH 5.0, centrifuged to remove excess liquid, and cooled at 0 °C. The inhibited enzyme sample was then applied to the column and spun for 1 min at 4000 rev./min in a Jouan model GR411 centrifuge at 0 °C. An Eppendorf tube containing 200 μ l of 10% TFA was used to collect the eluant. The function of the TFA was to denature the enzyme and trap the acyl-enzyme intermediate. It was necessary to add the acid after removal of excess substrate and product because the hydrolysed nafcillin precipitated out at low pH and adsorbed the protein. The eluant was then analysed by h.p.l.c.

A Beckman gradient h.p.l.c. system equipped with a model 165 detector and C3 ultrapore RPSC column was used to analyse the sample. A 0.1% TFA/acetonitrile gradient (5–45% acetonitrile in 15 min) was used. The eluant from h.p.l.c. was monitored at both 280 and 325 nm (the latter indicating the presence of the nafcillin chromophore). To ensure that there was enough sample for the peptide-fragmentation experiments, four such trapping experiments were done. The four samples were combined and concentrated to 250 μ l by using a microconcentrator [Centricon 10; 4 °C; centrifuged at 7000 rev./min for 45 min]. The concentrated sample was then further purified by h.p.l.c.

Peptide fragmentation. The concentrated sample from above was treated with pepsin (at a molar ratio pepsin/ β -lactamase of 1:10) at 20 °C. The digestion (at pH 2.5) was allowed to proceed for 3 h. H.p.l.c. was used to separate and collect the peptides which carried the bound substrate. A multiple injection scheme was used to introduce the sample to the h.p.l.c. column. Separation of the peptides with bound nafcillin was achieved on a C3 ultrapore RPSC column with the following gradient elution system. Solvent A was water containing TFA (0.1%). Solvent B was acetonitrile containing TFA (0.1%). The gradient began at 5% solvent B. After 2 min (the sample was injected during this period of time) a linear ramp was used (over 30 min) to 45% solvent B. After a further 5 min the gradient was decreased to 5% solvent over 18 min. The eluant from the h.p.l.c. was monitored at 325 and 220 nm. The peptides with bound substrate were collected. The labelled fractions were combined and dried under a stream of N₂.

The dried sample was dissolved in 160 μ l of 50 mM-phosphate buffer, pH 7.8, and subjected to trypsin digestion (trypsin/ β -lactamase molar ratio 1:10) at 20 °C. The digestion was allowed

to proceed for 3 h. H.p.l.c. was again employed to separate and to collect the peptide with the bound substrate. The method used to collect the labelled peptide was the same as that used in the pepsin digestion.

Amino acid sequence. The labelled peptide isolated from the trypsin digestion was sequenced by Edman degradation on a MilliGen/Biosearch 6600 ProSequencer.

RESULTS AND DISCUSSION

Direct observation of the enzymic hydrolysis of nafcillin

The difference spectrum of unhydrolysed and hydrolysed nafcillin (inset to Fig. 1) shows that the maximum $\Delta\epsilon$ is in the vicinity of 325–330 nm. In order to determine whether nafcillin is a normal substrate or a mechanism-based inhibitor (i.e. type A substrate) for the staphylococcal β -lactamase, we monitored the hydrolysis of nafcillin at 325 nm at 20 °C. The results are shown in Fig. 1, and were best fitted by a first-order process followed by a zero-order reaction. Significantly, only a small amount of the nafcillin was hydrolysed at the completion of the first-order process, indicating inhibition of the enzyme. Thus both the kinetics and amplitude change are consistent with the behaviour expected for a type A substrate. The rate of the initial exponential decrease in A_{325} was $2.6 \times 10^{-2} \text{ s}^{-1}$, and corresponds to the rate of inactivation of the enzyme, assuming that substrate-saturation conditions obtained. The subsequent very slow zero-order reaction, with rate $4.1 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$, would correspond to the steady-state turnover reaction, resulting from whatever amount of non-inhibited enzyme was present. The rate of the initial phase was in good agreement with the rate of inactivation determined independently (see below). Only 15% of the nafcillin was hydrolysed after 1000 s when the enzyme-catalysed reaction appeared to be complete. On the basis of these results it is concluded that nafcillin is a type A substrate for the staphylococcal enzyme and that it takes approx. 200 s to inactivate the enzyme. Moreover, to confirm that the levelling off of the hydrolysis was not due to depletion of substrate but due to the enzyme being completely converted into the transiently inhibited form, a fresh sample of enzyme was added to the incubation mixture after 1000 s. The hydrolysis pattern observed in Fig. 1 was again observed.

Fig. 1. Direct observation of nafcillin inactivation

The hydrolysis of nafcillin was monitored by the absorbance change at 325 nm. The concentration of nafcillin was 84 mM, and the concentration of the enzyme was 7 μ M. The solid curve represents a first-order reaction of rate constant $2.6 \times 10^{-2} \text{ s}^{-1}$, followed by a zero-order reaction of rate $4.1 \times 10^{-3} \text{ M} \cdot \text{s}^{-1}$. The inset shows the u.v.-visible difference spectrum of hydrolysed and unhydrolysed nafcillin. The hydrolysed nafcillin sample was prepared by incubating 0.68 μ M- β -lactamase with 0.4 mM-nafcillin.

Fig. 2. Time course of inactivation

β -Lactamase ($7 \mu\text{M}$ final concn.) was incubated in a 100 mM -nafcillin solution at $\text{pH } 7.0$ and at 20°C . Aliquots from the incubation were diluted 1000-fold, then assayed with benzylpenicillin (2.2 mM).

Time-dependent inactivation

In order to confirm that nafcillin behaved as both a substrate and an inhibitor, aliquots were removed from a mixture of β -lactamase and nafcillin and assayed with benzylpenicillin. Fig. 2 shows that progressive inactivation of the enzyme occurs as a function of time. Since, as is shown below, the inactivation is transient, this is evidence that nafcillin is a type A inhibitor (Citri *et al.*, 1976; Pain & Virden, 1979). These results show that essentially complete inactivation occurred in 200 s under the experimental conditions used. These data also enable a comparison of the rate of inactivation to the rates observed in the experiment of Fig. 1 to be made. The rate of inactivation determined from the data of Fig. 2 is $2.9 \times 10^{-2} \text{ s}^{-1}$, which agrees very well with the higher rate constant obtained from the direct observation study of $2.6 \times 10^{-2} \text{ s}^{-1}$.

Effect of inhibitor concentration on inactivation

As shown in Fig. 3 the amount of enzyme inactivated is a function of the nafcillin concentration. The data fit a saturation binding curve, with a K_d value of 33 mM . At concentrations greater than 80 mM , nafcillin completely inactivated staphylococcal β -lactamase. The result of this experiment also provided information on the concentration of nafcillin to be used in the trapping experiment.

Re-activation of inactivated enzyme

When an aliquot of completely inactivated enzyme was diluted into a solution of benzylpenicillin and the reaction monitored for

Fig. 3. Effect of nafcillin concentration on inactivation

The inactivation was done at $\text{pH } 7.0$ and at 20°C with $5 \mu\text{M}$ -enzyme.

Fig. 4. Re-activation of nafcillin-inactivated β -lactamase, monitored with benzylpenicillin

Assay conditions were $\text{pH } 7.0$ and 20°C . The solid line represents the re-activation assay, the broken line is the control (no incubation with nafcillin). The latter has been offset to start at the same absorbance as the re-activation assay. A 0.4 cm path length was used to minimize background absorbance.

an extended time period, it was observed that not only did the enzyme re-activate, but that complete recovery of the enzyme activity was obtained (Fig. 4). The rate of re-activation was calculated to be $1.9 \times 10^{-3} \text{ s}^{-1}$. In such a re-activation study it is important to have excess substrate, in this case benzylpenicillin, and low enzyme concentrations, in order to ensure that the enzyme is fully re-activated before the substrate concentration decreases to below saturation. If these conditions are not met, erroneous values of the rate and amount of re-activation may be obtained (Koerber & Fink, 1987).

Trapping the acyl-enzyme intermediate

Since nafcillin has a significant absorbance at 325 nm , whereas β -lactamase does not, one can use the nafcillin chromophore as a label in trapping experiments. Thus β -lactamase was incubated with excess nafcillin until inactivation was complete, the pH was then lowered to 5 to slow the re-activation reaction, and the incubation mixture passed through a centrifugal gel-filtration column at 0°C to separate the enzyme from excess nafcillin and products. The eluant was then analysed by h.p.l.c. under denaturing conditions. Thus only covalently bound nafcillin would be co-eluted with the enzyme fraction. Fig. 5(a) shows that the enzyme is covalently labelled with nafcillin and also indicates the retention times for the excess substrate (7.43 min), the product(s) (9.8 and 11.5 min) and the enzyme-substrate intermediate (13.3 min). From the molar absorption coefficient of nafcillin at 320 nm and at $\text{pH } 2.0$, which is $3000 \text{ M}^{-1} \cdot \text{cm}^{-1}$, one can calculate the stoichiometry of the labelled enzyme, which was found to be $0.8:1$.

The eluant fraction corresponding to the enzyme fraction was collected from four runs, combined and concentrated to approx. $250 \mu\text{l}$ by means of a microconcentrator. The concentrated sample was then again chromatographed by h.p.l.c.; the results are shown in Fig. 5(b). The shoulder in the enzyme peak observed at 280 nm reflects the partial resolution of the free enzyme from that labelled with nafcillin. In a separate acyl-enzyme trapping experiment, in which the incubation of enzyme with nafcillin was stopped after 1 min (which resulted in a 50:50 mixture of inhibited and free enzyme), the h.p.l.c. trace clearly showed two resolved forms of enzyme corresponding to the free and the inhibited enzyme.

The fractions containing the labelled enzyme were collected and subjected to pepsin digestion. As shown in Fig. 6, three

Fig. 5. H.p.l.c. traces of nafcillin-labelled β -lactamase

Elution profiles B and D are at 325 nm, those for A and C at 280 nm. Profiles B and D are for a typical nafcillin-trapping experiment after the centrifugal minigel chromatography. H.p.l.c. traces A and C show the profile of the concentrated and purified sample after combining the labelled enzyme fractions from four trapping experiments. The enzyme-substrate complex is eluted at 13.3 min, and the substrate and product(s) at 6.52 and 9.15 min, respectively. Trace B is offset by 0.15 from trace A, whereas trace D is offset by 0.01 from trace C.

Fig. 6. H.p.l.c. traces of nafcillin-labelled peptides

The upper trace is at 325 nm and the lower one at 215 nm. This Figure shows the elution profile after partial pepsin digestion. The peaks with retention times at 8.7, 10.8 and 11.2 min slowly converted into the peak at 8.9 min.

Fig. 7. H.p.l.c. traces of nafcillin-labelled peptide after trypsin digestion of the labelled fractions from the pepsin digestion

The upper trace is at 325 nm and the lower one at 215 nm. A single labelled peptide with retention time of 7.2 min was observed.

major labelled peaks were observed, which with increasing time of digestion collapsed into a single peak with retention time 9.8 min. The labelled peptides were collected and then subjected to trypsin digestion. A single labelled peptide was collected (Fig. 7). The sequence of this labelled peptide was Ala-Tyr-Ala-Ser-Thr-Ser-Lys. This corresponds to the region from A67 to K73. On the basis of previous work pointing to the central role of Ser-70, we conclude that nafcillin forms an acyl-enzyme intermediate with β -lactamase via Ser-70.

Mechanistic implications

The explanation for the inhibition of β -lactamase by type A substrates is still unclear (Kiener & Waley, 1977; Pain & Virden, 1979; Kiener *et al.*, 1980; Persaud *et al.*, 1986; Fink *et al.*, 1987; Hardy & Kirsch, 1989). Two possible sources of inhibition have been postulated; a substrate-induced conformational change or a chemical effect due to acylation at a residue other than the normal active-site serine residue. Kinetic studies have shown that the simplest kinetic scheme to account for the observations is that shown in eqn. 1 (Kiener *et al.*, 1980; Frere, 1981; Fink *et al.*, 1987):

The species EA^* differs from the normal acyl-enzyme (EA) in that it is hydrolysed very slowly. The results of the present investigation indicate that EA^* must be a conformational variant of the normal acyl-enzyme rather than one involving a different site of acylation. We conclude that type A substrates react with the class A β -lactamases by acylation of the same serine residue as normal substrates. However, the bulky hydrophobic side chains, which characterize the type A substrates, cause a con-

formational change which results in a very low rate of deacylation, and is thus responsible for their transient inhibition.

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