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Unexpected influence of ionic strength on branched-pathway interactions between β-lactamases and β-halogenopenicillanates

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Ionic strength strongly influenced the turnover/inactivation ratio in the interaction between β-halogenopenicillanates and some class A β-lactamases. This suggested the stabilization of a highly charged intermediate by solvation. Those data could be interpreted on the basis of a reaction pathway where an episulphonium ion was transiently formed. The various mechanisms proposed for explaining the formation of the dihydrothiazine chromophore are discussed.

INTRODUCTION

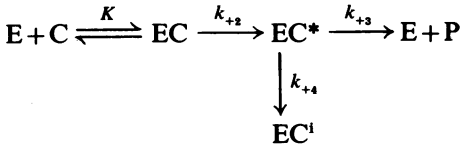
Inactivation of active-serine β-lactamases by halogenopenicillanates (Pratt & Loosemore, 1978; Orlek *et al.*, 1980) is accompanied by a rearrangement of the penicilloyl moiety of the acyl-enzyme into a dihydrothiazine chromophore (EC^i, Scheme 1). With some class A enzymes, hydrolysis of the acyl-enzyme sometimes competes with that rearrangement (Scheme 1, $k_{+3} \neq 0$) and the product itself readily rearranges into 2,3-dihydro-2,2-dimethyl-1,4-thiazine-3,6-dicarboxylate (Frère *et al.*, 1982; De Meester *et al.*, 1986). Branched pathways have not been detected with class C enzymes, and the Zn^{2+}-containing β-lactamases utilize β-halogenopenicillanates as normal substrates (Bicknell *et al.*, 1985; De Meester *et al.*, 1986).

The various mechanisms that have been proposed (Cohen & Pratt, 1980; Charnas & Knowles, 1981; Foulds *et al.*, 1984) to account for the formation of the dihydrothiazine fail to explain the following experimental results.

(1) No spontaneous opening of the thiazolidine ring has been observed at neutral pH with long-lived acyl-enzyme adducts formed between penicillins and active-site-serine DD-peptidases (Frère *et al.*, 1976, 1978).

(2) When turnover of the inactivator was observed, the product rapidly rearranged into the dihydrothiazine (Frère *et al.*, 1982*a*), which makes it difficult to involve an enzyme-linked base in the rearrangement.

We now wish to report that the drastic effects of ionic strength on the apparent turnover/inactivation ratio suggest a different rearrangement pathway, possibly one involving an episulphonium intermediate.



Scheme 1. Interaction between β-lactamases (E) and β-halogenopenicillanate (C).

In many cases, the k_{+3} step is not detected.

MATERIAL AND METHODS

Clavulanic acid, β-iodo-, β-bromo- and β-chloropenicillanic acids were gifts of Dr. G. Rolinson (Beecham Pharmaceuticals, Betchworth, Surrey, U.K.), Dr. J. Kemp (Pfizer Central Research, Sandwich, Kent, U.K.), Dr. H. Vanderhaeghe (Katholiek Universiteit, Leuven, Belgium) and Dr. M. Page (The Polytechnic, Huddersfield, West Yorkshire, U.K.) respectively. Details of synthesis of β-iodopenicillanic acid sulphone will be provided by J. M. F. on request. The β-lactamases of *Streptomyces albus* G, *Klebsiella pneumoniae* 10436, *Bacillus licheniformis* 749C and *Bacillus cereus* 5/B6 were purified as described by De Meester *et al.* (1987*a*), Joris *et al.* (1987), Thatcher (1975*a*) and Thatcher (1975*b*) respectively. The RTEM-2 β-lactamase was purchased from the Experimental Plant Microbial Technology Laboratory, Porton Down, Salisbury, Wiltshire, U.K. The DD-peptidase of *Actinomadura* R39 was purified as described by Frère *et al.* (1974).

The conductivity of the buffer (sodium phosphate, pH 7.0) was adjusted to various values as measured with a Metrohm E527 conductometer equipped with a cell the constant of which was 11.6 cm^{-1}. Under those conditions, 50 mM-sodium phosphate buffer (pH 7.0) and 50 mM-NaCl yielded conductance values of 0.45 and 0.40 mS respectively. Variations of ionic strength were always measured as variations of conductivity. All experiments were performed in the presence of EDTA (10 μM) to inactivate any contaminating trace of β-lactamase II. The presence of EDTA did not alter the properties of the class A β-lactamases. In the case of the *Streptomyces albus* G β-lactamase, all buffers contained 5% (v/v) ethylene glycol and 5% (v/v) glycerol.

Kinetic measurements

Inactivation rate constants (k_i) were measured in the presence of a reporter substrate by continuously monitoring the rate of hydrolysis of nitrocefin or cephaloridin. Those experiments were performed with a Beckman DU8 spectrophotometer linked to an Apple II microcomputer via a RS232C interface (De Meester *et al.* 1987*b*). The value of the ratios $(k_i)_{lim.}/K_m$ and k_{+2}/K were computed as described previously (Frère *et al.*, 1982*b*). The apparent $(k_{+3}+k_{+4})/k_{+4}$ ratio was

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determined by measuring the minimum C_0/E_0 ratio necessary to obtain complete inactivation of the enzyme (where E_0 and C_0 are the initial concentrations of enzyme and inactivator respectively). The errors (S.D.) on the measurements of $(k_i)_{lim.}/K_m$ and of $(k_{+3}+k_{+4})/k_{+4}$ were about 5 and 10% respectively. In the following discussion it will be useful to remember that, in the case of branched pathways and if k_{+3} is $\gg k_{+4}$, inactivation is characterized by a pseudo-first-order rate constant:

$$k_i = \frac{(k_i)_{lim.}[C]}{[C]+K_m^C}$$

where

$$(k_i)_{lim.} = \frac{k_{+2}k_{+4}}{k_{+2}+k_{+3}+k_{+4}}$$

and

$$K_m^C = \frac{k_{+3}K}{k_{+2}+k_{+3}}$$

and thus

$$\frac{(k_i)_{lim.}}{K_m^C} = \frac{k_{+2}}{K}\cdot\frac{k_{+4}}{k_{+3}}$$

The stopped-flow experiments were performed on a Sigma ZWS-11 stopped-flow mixing unit (Biochem, Munich, Germany) adapted to a Dia-Log optical and detection set-up (Garching Instrument, Düsseldorf, Germany). The signals were analysed by a data-acquisition and treatment system for fast transient optical signals as described by Houssier & O'Kinski (1981).

RESULTS AND DISCUSSION

Influence of ionic strength on the apparent $(k_{+3}+k_{+4})/k_{+4}$ ratio

As shown in Fig. 1, increasing the ionic strength induced a dramatic decrease in the $(k_{+3}+k_{+4})/k_{+4}$ ratio for the three well-characterized class A enzymes exhibiting a branched pathway in their interaction with β-iodopenicillanate. Between 0.1 and 5 mS, the ratio decreased 80, 25 and 15-fold with the *S. albus* G, *K. pneumoniae* and RTEM enzymes respectively. The same Figure also shows that, with the RTEM β-lactamase, the nature of the cation appeared to have little influence and that the shape of the curve depended on the size of the anion rather than on its nucleophilicity. Those results could be explained by an increase of k_{+4} or a decrease of k_{+3}.

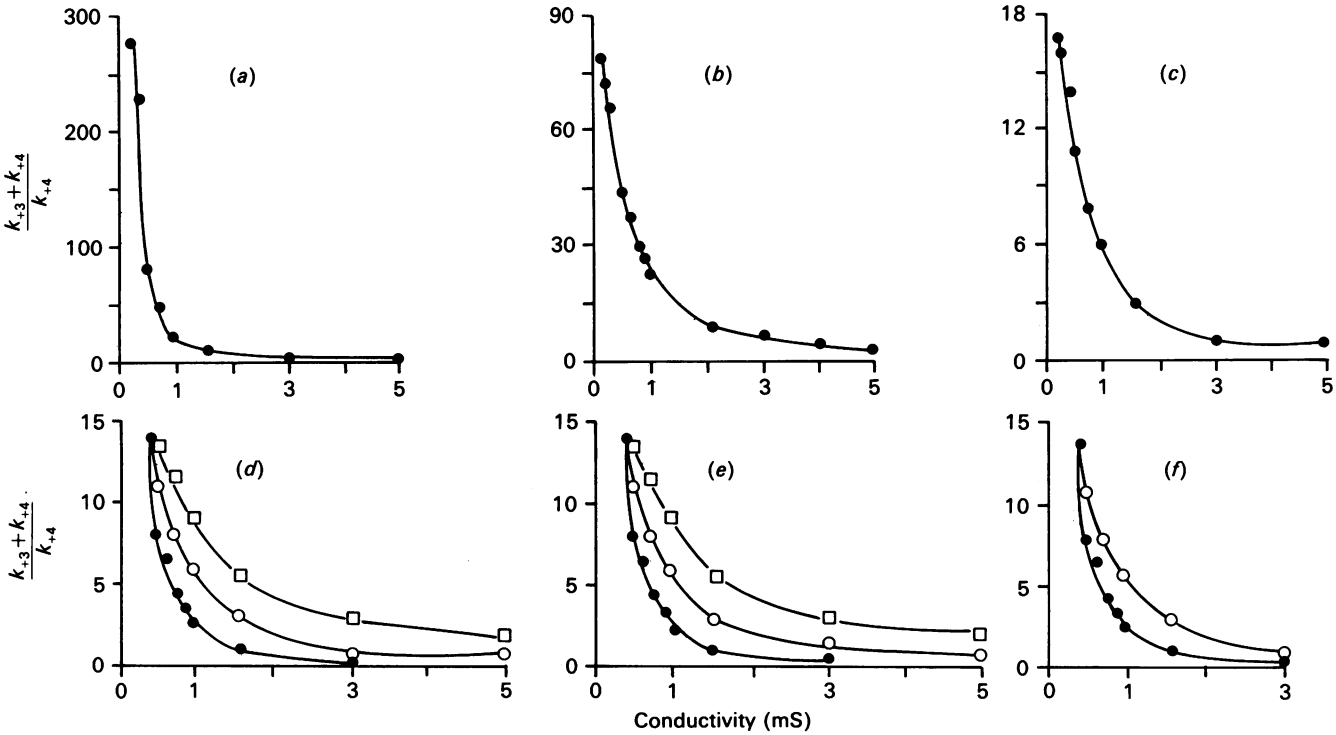


Fig. 1. Influence of ionic strength on the apparent $(k_{+3}+k_{+4})/k_{+4}$ ratio for the interaction between β-iodopenicillanate and the class A β-lactamases

(*a–c*) All experiments were performed at 30 °C in 6 mM-phosphate. The conductivity was adjusted to higher values by using NaCl. The apparent $(k_{+3}+k_{+4})/k_{+4}$ ratio was determined as explained in the Materials and methods section. (*a*) *S. albus* G β-lactamase: 10 μl samples of enzyme (1 mg·ml^{-1}) were incubated for 20 min at 30 °C with 10 μl of β-iodopenicillanate solutions of increasing concentrations. The presence of residual activity was detected by addition of 150 μM-nitrocefin in 50 ml of 100 μM-sodium phosphate, pH 7.0. (*b*) *Klebsiella pneumoniae* (K1) β-lactamase: conditions were as described above; the enzyme concentration was 0.5 mg·ml^{-1}. (*c*) RTEM β-lactamase: conditions were as described above; the β-lactamase concentration was 0.8 mg·ml^{-1}. (*d–f*) Influence of the nature of the salt: conditions were as in (*c*), and the following salts were used. (*d*) □, NaF; ○, NaCl; ●, NaI. (*e*) □, NaF; ○, $NaBF_4$; ●, NaI. (*f*) ●, NaI; ○, NaCl and LiCl (the curves were superimposable). The following conductivities were observed with 100 mM concentrations of the various salts: 0.58 mS (NaF), 0.8 mS (NaCl), 0.78 mS (NaI), 0.74 mS ($NaBF_4$) and 0.64 mS (LiCl). Above 2.0 mS, conductivities were no longer strictly proportional to concentrations.

Generally, little influence of ionic strength on the kinetic parameters of class A β-lactamases has been observed. Some authors routinely use 0.5 M-NaCl in incubation mixtures, and their results are not significantly different from those obtained at much lower salt concentrations. Moreover, Fukagawa *et al.* (1984) observed a monotonic and non-spectacular effect of ionic strength on the kinetic parameters of the *B. cereus* β-lactamase I with benzylpenicillin, ampicillin and cephaloridin.

Recent results obtained with the same enzyme (S. Waley, personal communication) indicate that the values of k_{+2} and k_{+3} are similar with several penicillins. In that case, variations of k_{+3} due to ionic strength would thus directly reflect on the $k_{cat.}$ values. With the *S. albus* G enzyme, the addition of 0.6 M-NaCl to 10 mM-sodium phosphate *decreased* the $k_{cat.}$ value by a factor of 3 with nitrocefin and 5 with cephaloridin. Moreover, the study of other branched-pathway inactivators with the three enzymes studied in the present work yielded the following results.

(i) Clavulanate inactivated the *S. albus* G β-lactamase with a turnover/inactivation ratio of 18000±1000 in 50 mM-sodium phosphate, pH 7.0 (Frère *et al.*, 1982*b*). The addition of 1 M-NaCl did not alter that ratio.

(ii) β-Iodopenicillanic sulphone inactivated the *K. pneumoniae* β-lactamase with turnover/inactivation ratios of 750, 850, 1000 and 2500 in 15 mM-sodium buffer added with 0, 40, 100 and 350 mM-NaCl respectively.

Attempts to obtain an estimation of k_{+4} were made with the *B. licheniformis* β-lactamase for which no turnover of β-iodopenicillanate was observed (De Meester *et al.*, 1986). Those experiments, performed with the help of the stopped-flow equipment, indicated that the appearance of the enzyme-bound chromophore at 320 nm remained strictly pseudo-first-order: at β-iodopenicillanate concentrations of 5, 50 and 500 μM and in 10 mM-sodium phosphate, pH 7.0, the values of the characteristic constants were 0.15±0.05, 1.5±0.3 and 15±5 s^{-1} respectively. That the apparent pseudo-first-order rate constant remained proportional to the β-iodopenicillanate concentration ($k_i/[C] = k_{+2}/K = 30000\ M^{-1}\cdot s^{-1}$) and very similar to that obtained by monitoring the inactivation of the enzyme (De Meester *et al.*, 1986), as well as the absence of lag in the appearance of the chromophore, indicated that the rate of formation of the acyl-enzyme was in all cases smaller than the rate of the rearrangement. It could thus be concluded that k_{+4} was much larger than 15 s^{-1}, but it was not possible to directly measure the influence of the ionic strength on the value of k_{+4}.

Variation of the inactivation rate with ionic strength

The pseudo-first-order inactivation rate constant for the inactivation of the K1 β-lactamase was measured at a high C_0/E_0 ratio so that the hydrolysis of the inactivator remained negligible. To 500 μl of 70 μM-nitrocefin in 6 mM-sodium phosphate, pH 7.0, added with various NaCl concentrations and containing β-iodopenicillanate concentrations varying from 20.5 to 82.2 μM (20.5, 32.9, 54.8 and 82.2), 50 ng of enzyme were added and the absorbance was continuously monitored at 482 nm. At each ionic strength, the apparent first-order rate constant remained proportional to the inactivator concentration and only the $(k_i)_{lim.}/K_m$ ratio could be measured (Frère *et al.*, 1982*b*). The value of k_{+2}/K was also computed for each ionic strength. Table 1 shows that the $(k_i)_{lim.}/K_m$ ratio increased with ionic strength, whereas the k_{+2}/K ratio slightly decreased. Those results could again be explained by an increase of k_{+4} which more than compensated for the decrease of k_{+2}/K.

Table 1. Influence of ionic strength (expressed as conductivity, μ) on the values of the $(k_i)_{lim.}/K_m$, $(k_{+3}+k_{+4})/k_{+4}$ and k_{+2}/K ratios for the inactivation of the K1 β-lactamase by β-iodopenicillanate

μ (mS)	$(k_i)_{lim.}/K_m$ ($M^{-1}\cdot s^{-1}$)	$\frac{k_{+3}+k_{+4}}{k_{+4}}$	k_{+2}/K ($M^{-1}\cdot s^{-1}$)
0.38	9.7×10^4	65	6.2×10^6
1.54	3×10^5	12	3.3×10^6
2.55	3.7×10^5	7	2.2×10^6
3.62	4.5×10^5	5	1.8×10^6
4.4	5.8×10^5	4	1.7×10^6

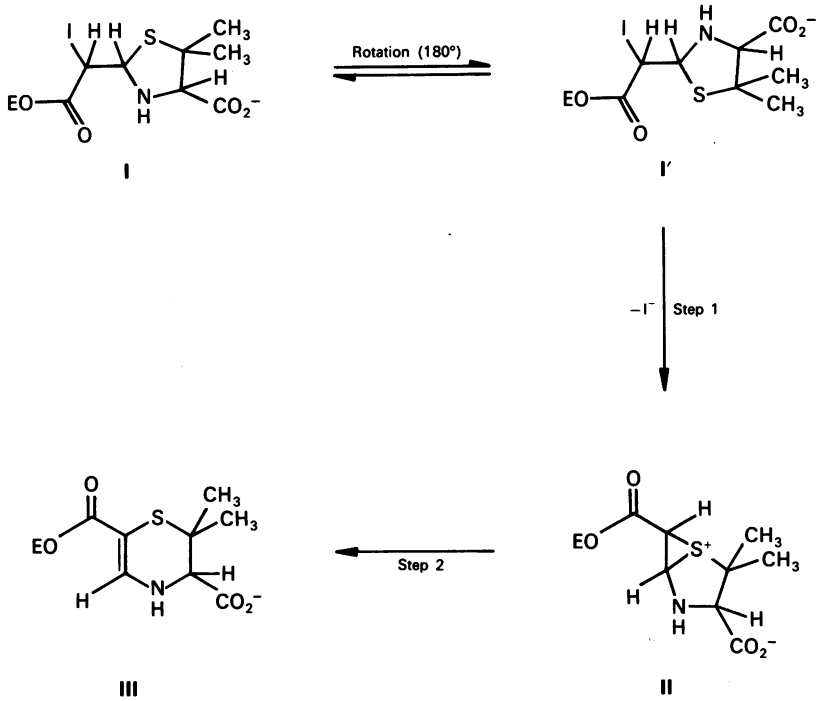
Influence of the nature of the halogen

At pH 7.0 and in 6 mM-sodium phosphate, the *K. pneumoniae* enzyme exhibited $(k_{+3}+k_{+4})/k_{+4}$ ratios of 85 and 875 with β-iodo- and bromo-penicillanate respectively. With β-chloropenicillanate, although the $(k_{+3}+k_{+4})/k_{+4}$ ratio was decreased by increasing the buffer concentration to 50 mM, it was still larger than 3000. This decrease of the ratio with increasing leaving capacity of the halogen could be interpreted by assuming that the departure of the halogen was the rate-limiting step of the rearrangement. Moreover, with β-chloropenicillanate, a branched pathway was also observed with the *B. licheniformis* β-lactamase. In 50 mM-sodium phosphate, pH 7.0, the $(k_{+3}+k_{+4})/k_{+4}$ ratio was 1000 and $(k_i)_{lim.}/K_m$ was 350 $M^{-1}\cdot s^{-1}$.

Those results were also in good agreement with a decrease of the value of k_{+4} when I is replaced by Cl. Indeed, if the difference was only due to an increased k_{+3}, a rather unlikely value much larger than 15000 s^{-1} should be assumed for the β-chloro derivative.

Proposed rearrangement pathway

A plausible rearrangement pathway must account for the very rapid rearrangement and for the unexpected influence of ionic strength on the $(k_{+3}+k_{+4})/k_{+4}$ ratio, which suggested the stabilization of a highly charged intermediate by a solvation effect. Since the pathways proposed previously (Cohen & Pratt, 1980; Charnas & Knowles, 1981; Foulds *et al.*, 1984) involved the formation of a thiolate anion, it was important to obtain further information allowing their rejection. That the base-catalysed removal of the C^6 proton was quite unlikely was shown by the facts that the dihydrothiazine was quantitatively and rapidly formed upon methanolysis of β-iodopenicillanic acid and that the dihydrothiazine was also immediately formed when the TEM and *Klebsiella* enzymes hydrolysed β-iodopenicillanate at pH 5.0. The chromophore also appeared without any delay upon the hydrolysis of 500 μl of 100 μM-β-iodopenicillanate by 30 μg of the *Bacillus cereus* Zn^{2+} β-lactamase II in 100 μM-cacodylate buffer, pH 4.75, containing 50 μM-$ZnSO_4$. Conversely, no indication of



Scheme 2. Proposed episulphonium pathway for the formation of the dihydrothiazine chromophore

I, Acyl enzyme; I', acyl enzyme after a 180° rotation around C^5–C^6; II, episulphonium intermediate; III, rearranged enzyme-linked dihydrothiazine.

the presence of a free thiol group was obtained by addition of 5,5'-dithiobis-(2-nitrobenzoic acid) to (1) 100 μM-α-methylbenzylpenicilloate at pH 7.0 or (2) 13 μM-benzylpenicilloyl-enzyme (R39 DD-peptidase) at pH 7.8. This indicated that, under those conditions, no spontaneous opening of the thiazolidine ring occurred at a detectable rate and that the rapid formation of the chromophore was impossible to explain on that basis. Moreover, the ionic-strength effect was specific to β-halogenopenicillanates. The mechanisms generally accepted for the branched pathways observed with clavulanate or penicillin sulphones also involve the breaking of the C^5–S or C^5–O bond, and little variation of the $(k_{+3}+k_{+4})/k_{+4}$ ratio was observed in those cases.

As an alternative hypothesis, we propose that the highly charged intermediate might be the episulphonium ion shown in Scheme 1. A similar episulphonium ion had already been envisaged by McMillan & Stoodley (1966) as an intermediate in the slow rearrangement of 4D-carbomethoxy-5,5-dimethyl-α-chloro-2-thiazolidine acetate to the dihydrothiazine, but rejected for kinetic reasons.

In the model proposed here (Scheme 2), the departure of the halogen (Step 1) would be rate-limiting. This would also explain the observation that the rearrangement which follows the hydrolysis of the β-lactam amide bond is several orders of magnitude faster with β-halogenopenicillanates than with the α isomers (Pratt & Cahn, 1988). Similarly, with the β-chloro compound, we observed an immediate rearrangement, whereas McMillan & Stoodley (1966) isolated a rather stable product by methanolysis of methyl-6α-chloropenicillanate. Indeed, molecular models of the episulphonium ions obtained starting with the hydrolysis products of β-iodopenicillanate and α-iodopenicillanate clearly showed that the latter was so sterically hindered that its formation was impossible. Among the mechanisms proposed for explaining the rearrangement, that which involves an intermediate episulphonium ion remains the only one in agreement with all the experimental facts. It is interesting to remember that an episulphonium ion is also an intermediate in the reactions of mustard gas, which proceed orders-of-magnitude faster than expected for primary alkyl halides (Bartlett & Swain, 1949).

Although most of the evidence remains indirect, the high rate of the phenomenon seems to exclude the possibility of a more direct demonstration. Indeed, in all the experiments that were performed, the opening of the β-lactam amide (k_{+2} step) always appeared to remain rate-limiting.

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