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Substrate-Induced Deactivation of Penicillinases

STUDIES OF β-LACTAMASE I BY HYDROGEN EXCHANGE

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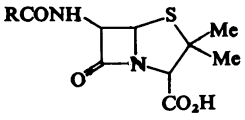
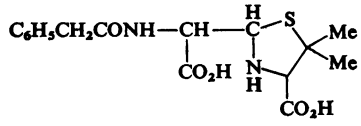
The conformational motility of β-lactamase I from *Bacillus cereus* was studied by hydrogen exchange. The time course of the isotopic replacement of peptide hydrogen atoms was followed by 'exchange-in' or 'exchange-out' experiments. Many of the substrates for this enzyme that have *o*-substituted aromatic or heterocyclic side chains (e.g. methicillin or cloxacillin) are known to effect a decrease in enzymic activity ('substrate-induced deactivation'). There was a marked discontinuity in the exchange-out curve when methicillin or cloxacillin was diffused into the enzyme solution. About one-half of the hydrogen atoms that were probed were affected by the presence of these substrates, and the change in the reactivity of the hydrogen atoms was also large. Substrates that do not bring about deactivation (benzylpenicillin and cephalosporin C) do not affect the hydrogen exchange, nor do reversible competitive inhibitors such as the penicilloic acid or penilloic acid. On the other hand, Zn^{2+} ions do affect the hydrogen exchange; their effect is similar to that of methicillin or cloxacillin.

For nearly 20 years it has been known that β-lactamases (penicillinase, EC 3.5.2.6) are partially or completely inactivated by some of their substrates (Citri & Garber, 1961; Crompton *et al.*, 1962; Gourevitch *et al.*, 1962; Depue *et al.*, 1964; Dyke, 1967; Csányi *et al.*, 1970; Virden *et al.*, 1975; Strom *et al.*, 1976). This is the phenomenon now called 'substrate-induced deactivation'. Sometimes the effect has been inferred from the shapes of progress curves during an assay, and sometimes from an enhanced rate of inactivation by various reagents, especially iodine. The enhanced rate of inactivation is ascribed to a change in conformation of the enzyme brought about by the substrate. Citri (1973) calls this the 'conformative response' of the enzyme. Direct information about changes of conformation in solution is provided by hydrogen exchange (Linderstrøm-Lang, 1955; Hvidt & Nielsen, 1966; Englander *et al.*, 1972). We now describe the application of this technique to the substrate-induced deactivation of





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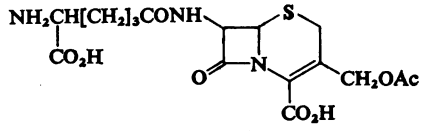

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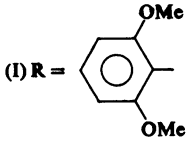

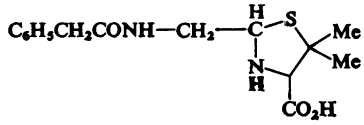

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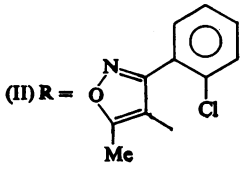

β-lactamase I from *Bacillus cereus*. This is an extracellular, monomeric, enzyme (Kuwabara & Abraham, 1967; Davies *et al.*, 1974*a*) and most of the sequence is known (Thatcher, 1975). Typical substrates that bring about deactivation of this enzyme are methicillin (I) and cloxacillin (II), and we have used these. Typical reversible competitive inhibitors are the penicilloic acid (III) and penilloic acid (IV) (P. A. Kiener & S. G. Waley, unpublished work) and we have also tested these. Cephalosporin C (V), a substrate that does not bring about deactivation (Citri & Zyk, 1965), has also been used.

Materials and Methods

Materials

β-Lactamase I was obtained by the method of Davies *et al.* (1974*a*), or by a modification (Kiener, 1976). Cephalexin was a gift from Eli Lilly, Indianapolis, IN, U.S.A. Cephalosporin C and benzylpenicillin were gifts from Glaxo Laboratories Ltd., Greenford, Middx., U.K., and methicillin and cloxacillin were gifts from Beecham Research Laboratories, Brockham Park, Surrey, U.K. ^3H_2O was from The Radiochemical Centre, Amersham, Bucks., U.K., and was diluted to 125mCi/ml before use.

Methods

^3H radioactivity was determined with a Nuclear-Chicago Unilux IIA liquid-scintillation counter. The scintillation fluid was as given previously (Browne & Waley, 1974); aqueous samples (50μl) were taken and added to 3ml of scintillation fluid. The samples were cooled to 4°C for 15min before they were counted for radioactivity; the efficiency for ^3H was about 25%. The following buffers were used (and are referred to below by their pH). At pH7.5, 18.6g of triethanolamine hydrochloride and 146.1g of NaCl, the pH being adjusted with NaOH and the volume made up to 5 litres; at pH6.6, 24.4g of $NaH_2PO_4,2H_2O$ and 33.5g of $Na_2HPO_4,12H_2O$ in 5 litres; an alternative buffer was from 16g of 3,3-dimethylglutaric acid and 146.1g of NaCl, the pH being adjusted with NaOH and the volume made up to 5 litres.

The course of exchange-in was followed after adding 50μl of ^3H_2O (125mCi/ml) to about 3mg of β-lactamase I in 1ml of pH7.5 buffer and keeping the solution at 37°C for 0.25–2h. Then the sample (except for a 50μl portion that was used to measure the specific radioactivity of the medium) was applied to a column (15cm×2.5cm diam.) of Sephadex G-25 (the column was twice as long when ligands were present) equilibrated with pH6.6 buffer, at 4°C. The column was eluted rapidly (600ml/h), and the protein was on the column for 3–8min (depending on the length of the column). Fractions (1ml) were collected, and the A_{280} was measured and portions were taken for determination of radioactivity. The A_{280} was taken as 1.00 for a 1mg/ml solution of β-lactamase I (Davies, 1974), and the mol.wt. was taken as 28000 (Davies *et al.*, 1974*a*).

Exchange-out experiments were carried out by first labelling the enzyme for 1h at pH7.5, 37°C, as described above, and then following the loss of radioactivity from the solution by hollow-fibre dialysis (Browne & Waley, 1973). Fractions from the Sephadex G-25 column ($A_{280}>0.1$) were combined, and 4ml was introduced into the fibres of a Bio-Fibre 50 beaker dialyser unit (Bio-Rad Laboratories Ltd., Bromley, Kent, U.K.) at 4°C, equilibrated with pH6.6 buffer. The volume of buffer outside the fibres was about 100ml, and the buffer was changed about nine times during the course of exchange-out. The effect of the addition of ligand was studied by replacing the usual buffer with buffer containing the ligand; after 10, 25 and 45min this buffer was replaced by fresh buffer containing ligand. Samples of the enzyme solution were taken for determination of radioactivity as previously described (Browne & Waley, 1973).

The extent of labelling was arrived at from:

$$\text{H atoms/molecule} = \frac{(\text{radioactivity of enzyme})/[\text{enzyme}]}{(1.2 \times \text{radioactivity of } ^3H_2O)/[^3H_2O]}$$

The concentration of the enzyme was obtained from the A_{280} as described above, and the concentration of ^3H_2O was taken as 110M; the factor of 1.2 is to allow for the equilibrium isotope effect for peptide H atoms (Englander & Poulsen, 1969).

The enzymic activity of β-lactamase I was determined with benzylpenicillin as substrate, at 30°C, in the presence of 0.5M-NaCl; a pH-stat or a spectrophotometer (Waley, 1974; Samuni, 1975) was used. The enzyme was regularly assayed after the hydrogen-exchange experiments to check that it had retained activity.

Results and Discussion

Course of exchange-in

The exchange-in took place at 37°C, pH7.5, and the protein was then separated from ^3H_2O at 4°C, pH6.6. Exposed peptide groups exchange about tenfold faster at 37°C than at 4°C and about tenfold faster at pH7.5 than at pH6.6, and have a half-life of a few seconds at 4°C, pH6.6 (Englander & Staley, 1969). These exposed peptide groups, which are on the surface and hydrogen-bond with the solvent, will be labelled during incubation but will lose the label during the separation from ^3H_2O. Those peptide groups that exchange about 100-fold more slowly will be detected; this is about the lower limit. The

upper limit is about 10^5-fold ($60\times60\times10\times10$), after incubation for 1h, 37°C, pH7.5, when the temperature and pH are taken into account, and also the difference between seconds and hours. Thus the hydrogen-exchange 'window' is here 10^2–10^4, and so, on the usual model (Englander *et al.*, 1972), we are examining regions of structure that are stabilized to the extent of 12–24kJ/mol (2.8–5.6kcal/mol). Most of the peptide H atoms in proteins are slowed for exchange by factors of 10^2 to perhaps 10^5 (Willumsen, 1971; Englander *et al.*, 1972), and so much of the structure should be probed in our experiments. The side-chain H atoms exchange at a rate too fast to be detected, but a small proportion of the H atoms found may represent the amide H atoms of asparagine and glutamine residues (which have half-lives of a few minutes at 4°C, pH6.6).

The course of exchange-in is shown in Fig. 1; the values given are means, derived from values for individual fractions from the column (about ten fractions) from at least two experiments. The value of about 135 H atoms was reached after 1h; prolonged incubation (96h) gave a value of 220 H atoms. The total number of peptide and amide H atoms is approx. 300 (Davies *et al.*, 1974*a*; Thatcher, 1975), so that a fairly small proportion of the peptide groups is exposed. Globular proteins usually have 20–40% of their peptide groups hydrogen-bonded to external solvent (Englander *et al.*, 1972; Willumsen, 1971; Downer & Englander, 1975).

We come now to the effects of ligands. The open-chain derivatives [penicilloates and penilloates, e.g. compounds (III) and (IV), the latter being decarboxylation products] are, in several cases, reversible competitive inhibitors of β-lactamase I (P. A. Kiener & S. G. Waley, unpublished work); they do not affect the course of the exchange-in (Fig. 1).

On the other hand, a substrate, cloxacillin (II), had a marked effect, both on the hydrogen exchange and on the activity. Much of the enzymic activity was lost within 15min when the solution contained 0.5M-cloxacillin, but the activity was regained during assay, and curves such as that shown in Fig. 4 of Davies *et al.* (1974*b*) were obtained. If the solution was diluted 200-fold before assay, full activity was obtained. After exchange-in in the presence of 0.5M-cloxacillin and passage down the Sephadex column the extent of labelling was very low (10 ± 3 atoms/molecule). These results suggest that the conformation of the protein is extensively, but reversibly, altered by this substrate; apparently the protein unfolded in high concentrations of cloxacillin and refolded when the concentration was lowered. The need for a high concentration of substrate to show these effects can be accounted for if an intermediate (or the enzyme–substrate complex) has a relatively low probability of

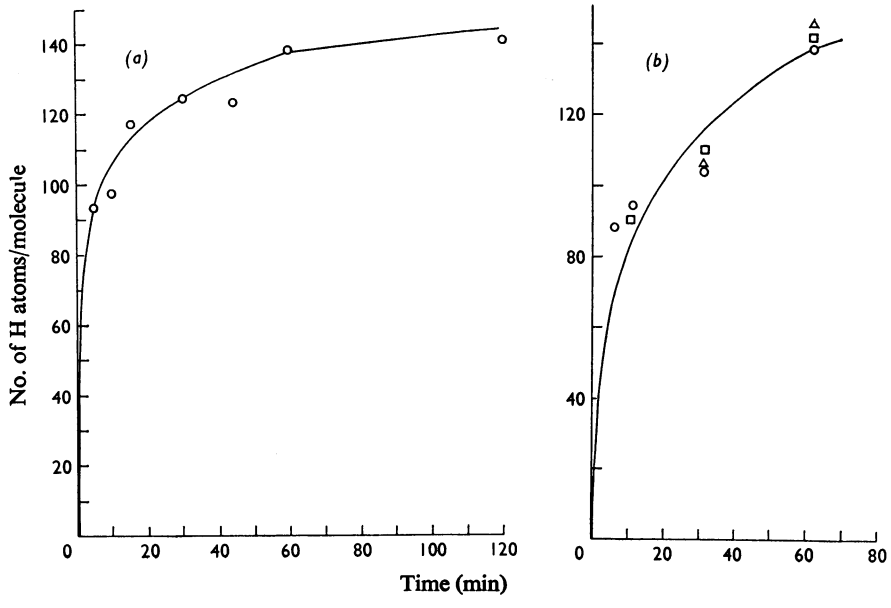

Fig. 1. *Exchange-in of β-lactamase I alone (a) and in the presence of ligands (b)*

Protein was incubated in triethanolamine hydrochloride buffer, pH7.5, containing ^3H_2O, for the time given on the abscissa, at 37°C, and then separated from the ^3H_2O on Sephadex G-25 at pH6.6, 4°C. In (*a*) the protein was unliganded (○); in (*b*) 10mM-penilloate from penicillin V (△), or 20mM-penilloate from benzylpenicillin (□), or 10mM-penilloate from methicillin (○), was present.

undergoing a change in conformation. Both the reversibility, and the speed, of these effects of cloxacillin on β-lactamase I distinguish them from those due to the non-specific reaction between penicillins and proteins described by Corran & Waley (1975); reversible conformational changes undergone by β-lactamase I are well known (Imsande *et al.*, 1970; Davies *et al.*, 1974*b*). Precipitation promptly occurred when 0.5M-methicillin and β-lactamase I were mixed at 37°C; this precluded further experiments.

The exchange-out procedure, in which the loss of ^3H from the protein is followed, is in many ways a more flexible procedure, and the results with this procedure are now described.

T.l.c. was carried out on silica-gel plates with acetone/acetic acid (19:1, v/v); compounds were detected by spraying with 1% $KMnO_4$.

Exchange-out

After exchange-in for 1h at 37°C, pH7.5, the course of exchange-out was followed at 4°C, pH6.6, by hollow-fibre dialysis as described in the Materials and Methods section. The curve (Fig. 2) can be roughly divided into two regions, as if there were about 65 H atoms exchanging with a half-life of about 20h and another 65 H atoms exchanging with a half-life of about 30min. The precise division is arbitrary, but this sort of distribution is common. The effects of ligands were examined after exchange-out had been proceeding for 4h; buffer lacking ligand was replaced by buffer containing ligand.

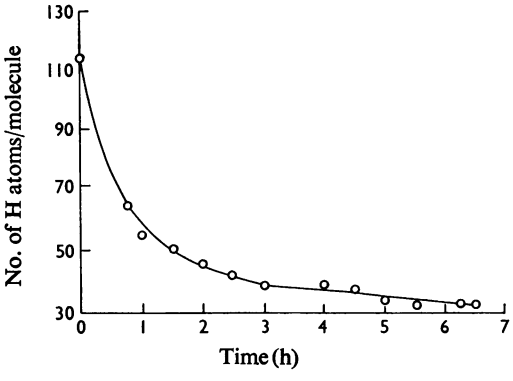

Fig. 2. *Exchange-out of β-lactamase I*
Protein was labelled by exchange-in for 1h at 37°C, pH7.5, and the protein was then separated from ^3H_2O on Sephadex G-25 and introduced into the fibres of the dialyser unit at 4°C, and the surrounding jacket filled with pH6.6 buffer. Samples of the solution containing protein were periodically removed for determination of radioactivity.

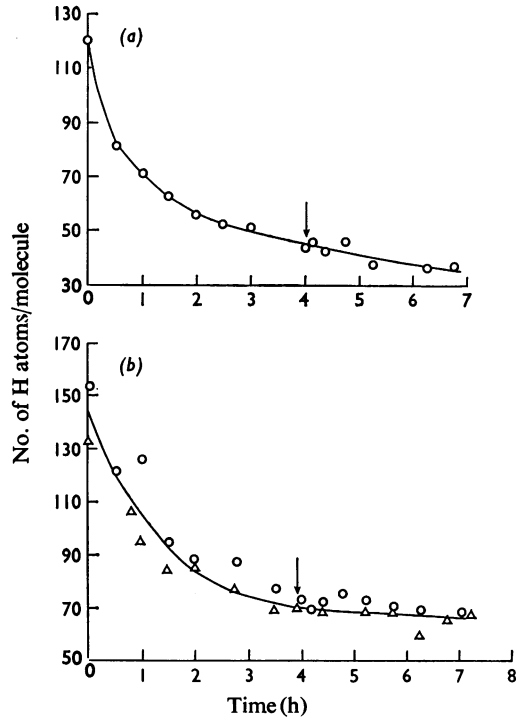

Fig. 3. *Effect of inhibitor and substrates on exchange-out*
Exchange-out was performed as described in the legend to Fig. 2; after 4h (arrow) the buffer in the jacket was replaced by buffer containing 20mM-penilloate from benzylpenicillin (*a*), or 3mM-benzylpenicillin (○) or 20mM-cephalosporin C (△) (*b*).

The penicilloates and penilloates did not affect the course of exchange-out (Fig. 3); this parallels the results on exchange-in. Hence combination with a ligand does not necessarily affect the conformation (as judged by the present technique). Nor does combination with a substrate, i.e. turnover, because neither benzylpenicillin nor cephalosporin C (V) caused a detectable change in conformation (Fig. 3). Nor did another cephalosporin, namely cephalexin (the 7-D-phenylglycyl derivative of deacetoxy-7-aminocephalosporanic acid). Fresh substrate was supplied in these experiments by frequent changes of buffer containing the substrate; the pH was also checked; it did not fall below 6.3.

Quite different results were obtained with methicillin or cloxacillin. There was an abrupt fall in the exchange-out curve when these substrates were introduced (Fig. 4). These conditions were 'milder' than in the exchange-in experiments; the concentration of methicillin or cloxacillin was much lower (10mM as opposed to 500mM) and the temperature was lower (4°C as opposed to 37°C). The enzyme

remained active, as judged by t.l.c. of samples of the buffer surrounding the fibres; the samples showed that substrate was being hydrolysed throughout the experiment. These results strikingly demonstrate that methicillin and cloxacillin, the compounds that bring about substrate-induced inactivation, also affect the course of hydrogen exchange. Their effects will now be analysed in more detail.

After exchange-out for 4h, there are about 70 H atoms remaining, nearly all with half-life about 20h. Methicillin or cloxacillin was introduced at 4h, and the curve fell (Fig. 4); the values of (H atoms)−(H atoms finally remaining) are plotted in Fig. 5. The final values, after about 7h, represent H atoms partly or entirely indifferent to the presence of methicillin or cloxacillin, whereas the difference plotted in Fig. 5 represents responsive H atoms. These responsive H atoms comprise, kinetically, one class (within the error of the experiments, about 3 H atoms), and have a half-life of about 45min. This half-life, and the number of responsive H atoms (about 35), were the same for methicillin and cloxacillin. Virtually all the H atoms with a half-life of 45min must be responsive H atoms, because indifferent H atoms with such a relatively short half-life would have been lost by 4h. Thus about one-half of the 70 H atoms remaining at 4h are responsive. Hence the change is by no means a local one. The responsive H atoms change from having a half-life of 20h to having a half-life of 45min. The analysis of Englander *et al.* (1972) ascribes such a change to a change in the free energy associated with the stabilization of segments of structure; the magnitude of the change is here $RT\ln(20/0.75)$, 12kJ/mol (3kcal/mol). As stressed by Englander *et al.* (1972), changes in stable structure (conformational changes) are bound to reveal themselves in changes of 'breathing' (segmental unfolding), and the change in 'breathing' transduces the change in conformation into a change in rate of H exchange. Our results thus provide direct evidence for a change in structure brought about by methicillin or cloxacillin. The change, moreover, was the same, whichever substrate was used. This suggests a reasonably well-defined characteristic transition.

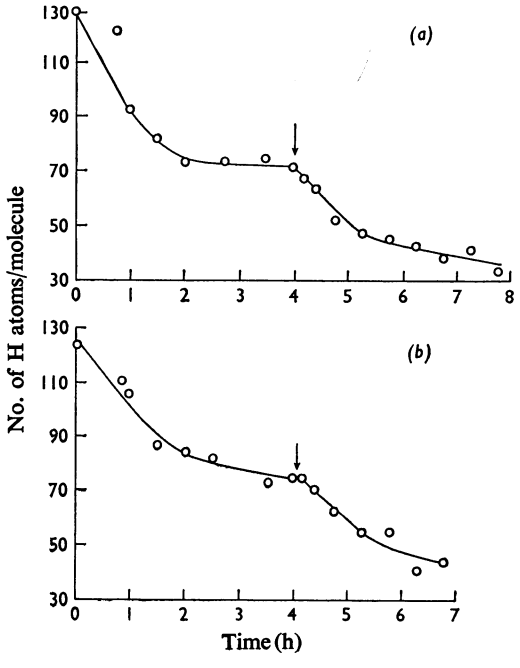

Fig. 4. *Effect of methicillin or cloxacillin on exchange-out*
Exchange-out was performed as described in the legend to Fig. 2. After 4h (arrow), 10mM-methicillin (*a*) or 10mM-cloxacillin (*b*) was dialysed in.

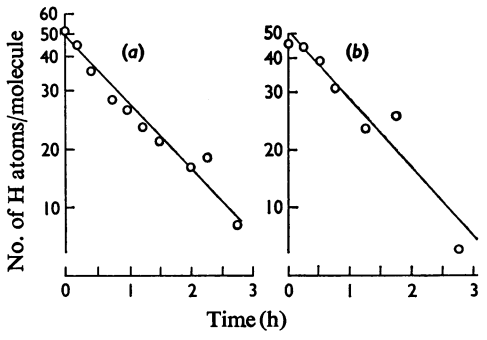

Fig. 5. *H atoms affected by dialysing in methicillin or cloxacillin*
The number of responsive H atoms was obtained from Fig. 4 by subtraction as described in the text; the time on the abscissa refers to the time after methicillin (*a*) or cloxacillin (*b*) was dialysed in.

Effects of Zn^{2+} ions on exchange-out

β-Lactamase II, the enzyme that accompanies β-lactamase I, requires Zn^{2+} for activity (Kuwabara & Abraham, 1967), but β-lactamase I does not, and in fact is often assayed in the presence of EDTA. Kuwabara (1970) reported that 3mM-Zn^{2+} (at pH7) inhibited β-lactamase I (by 95%), and D'Souza *et al.* (1975) obtained 40% inhibition at pH5.8 by 10mM-$ZnCl_2$. These results suggest that the extent of binding depends on the pH, and in accord with this we found 40% inhibition with 25mM-$ZnSO_4$ at pH5 (Kiener, 1976); it is, in fact, difficult to get reliable values for the extent of inhibition at higher pH values because of the appreciable non-enzymic hydrolysis of benzylpenicillin in the presence of Zn^{2+}.

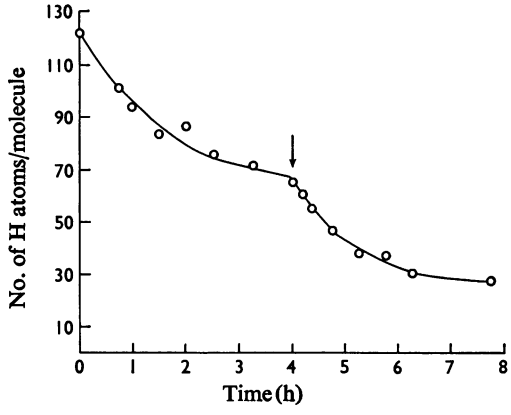

Fig. 6. *Effect of Zn^{2+} on exchange-out*
Exchange-out was described in the legend to Fig. 2; after 4h (arrow), 1mM-$ZnSO_4$ was dialysed in.

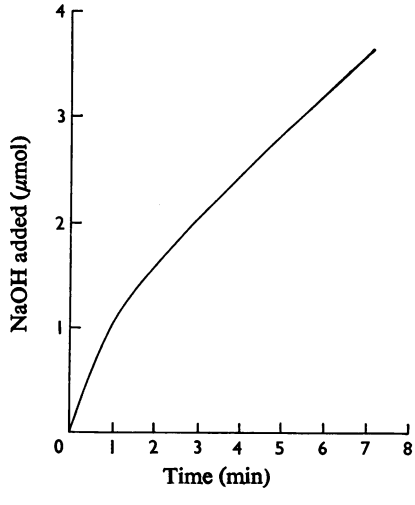

Fig. 7. *Slow transition in the hydrolysis of cloxacillin by β-lactamase I*
The course of the hydrolysis of 50mM-cloxacillin by 6μg of β-lactamase I at pH7, 4°C, was followed in the recording pH-stat.

The effect of Zn^{2+} on the exchange-out of β-lactamase I was examined by introducing 1mM-$ZnSO_4$ after 4h. The results (Fig. 6) show an abrupt change; the results with 10mM-$ZnSO_4$ were similar. The effects of Zn^{2+} are similar to those of methicillin or cloxacillin, and indeed there were about the same number of responsive H atoms, and they had the same half-life. Thus $ZnSO_4$ was having the same effect at pH6.6, 4°C, as the substrates, and, as far as can be told, bringing about the same change of conformation. High concentrations of salts [saturated $(NH_4)_2SO_4$] have been reported to convert the enzyme into a more iodine-sensitive form (Rudzik & Imsande, 1970); substrates such as methicillin and cloxacillin also enhance iodine-sensitivity (Citri & Zyk, 1965).

Inactivation or deactivation?

When there is a partial loss of enzymic activity the question arises as to whether some of the molecules are completely inactivated and some unchanged, or whether, on the other hand, all of the molecules have the same fractional activity. In the exchange-out experiments at 4°C the enzyme in the hollow-fibre dialyser was repeatedly exposed to fresh substrate. These conditions were favourable for the complete conversion of the enzyme, and activity was retained. Hence there was probably a change from one active conformation (or set of conformations) into another (less) active conformation (or set of conformations), i.e. deactivation. This change may be directly seen in the biphasic progress curve of an assay (Fig. 7). The transition from one form to another is relatively slow, and takes of the order of 1 min (Citri *et al.*, 1976).

The new, less-active, conformation is apparently more labile; it is more reactive towards iodine, and prone to thermal denaturation at 37°C. This instability towards heat is rather reminiscent of mutants where the replacement of one amino acid by another may lead to a less heat-stable enzyme. The unfolding at 37°C was evident in the exchange-in experiments.

Comparison of substrates

Citri & Zyk (1965) pointed out that it was those penicillins that were hydrolysed more slowly than 6-aminopenicillanic acid by β-lactamase I that brought about deactivation. Comparison of the structures of the substrates suggests that there may be an unfavourable interaction between part of the side chain of the penicillin and part of the protein, and, as a consequence, $k_{cat.}$ is lowered and the conformational response is evoked. The correlation between conformational change and kinetic parameter is with $k_{cat.}$ rather than with K_m, and neither products nor substrate analogues bring about deactivation, so that it may well be that it is an intermediate that undergoes the conformational change. Further work is needed to decide between this and possible alternative mechanisms.

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