Conformational Changes in the Extracellular β -Lactamase I from *Bacillus cereus* 569/H/9

By RICHARD B. DAVIES and E. P. ABRAHAM

Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.

and D. G. DALGLEISH*

Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford OX2 6HF, U.K.

(Received 30 May 1974)

1. The thermal denaturation and precipitation of β -lactamase I from *Bacillus cereus* 569/H/9 at 60°C are reversible, a soluble and almost fully active enzyme being obtained after solution of the precipitate in 5M-guanidinium chloride or 8M-urea and subsequent removal of the denaturing agent. 2. Inactivation of β -lactamase I occurs rapidly between 50° and 55°C and is shown by circular-dichroism spectra to be accompanied by an extensive conformational change. 3. A change to a different conformation occurs in 6M-urea. This change is also reversible; refolding with almost complete recovery of enzymic activity occurs within 5 min of dilution of the denaturing agent. 4. Inactivation of β -lactamase I at pH 3.0 and 11.0 is also associated with conformational changes, since a proportion of the lost activity is recovered within 5 min of adjustment of the pH to 7.0.

Several studies have been made of reversible conformational changes in β -lactamase I (α -penicillinase) from *Bacillus cereus* 569 and 569/H. Citri *et al.* (1960) stated that the α form of the exoenzyme was interconvertible with a form which resembled a cell-bound γ -penicillinase in its sensitivity to iodine. Rudzik & Imsande (1970) reported that the exoenzyme was changed in saturated $(\text{NH}_4)_2\text{SO}_4$ to an iodine-sensitive form and that it regained its original form when the $(\text{NH}_4)_2\text{SO}_4$ was removed. Imsande *et al.* (1970) showed that the denaturation of the exoenzyme in urea or guanidinium chloride was reversible.

Crompton *et al.* (1962) found that the β -lactamase I (penicillinase) from *B. cereus* 569 was much more susceptible to denaturation by heat than was the β -lactamase II (cephalosporinase) produced by the same organism. Kuwabara (1970) and Davies *et al.* (1974) made use of this finding to remove small amounts of β -lactamase I during the purification of β -lactamase II from *B. cereus* 569/H and 569/H/9 respectively. Davies *et al.* (1974) reported that the stability of β -lactamase I was higher than that of β -lactamase II at low pH values. The present paper describes further experiments relating to conformational changes in β -lactamase I and to their reversibility.

Materials and Methods

Materials

The β -lactamase I was a highly purified preparation corresponding to that obtained by Davies *et al.* (1974) at stage 3B. The stock enzyme solution was in the

'Tris- Zn^{2+} ' buffer, pH 7.0 (0.1 M with respect to Tris and 1 mM with respect to ZnSO_4) described by Davies *et al.* (1974), which had been used during the separation of the enzyme from β -lactamase II. Urea (AnalaR) and guanidinium chloride (AristaR) were from BDH Ltd., Poole, Dorset, U.K.

Methods

β -Lactamase activity. This was measured in a pH-stat as described by Davies *et al.* (1974) with benzylpenicillin as a substrate.

Circular-dichroism (c.d.) spectra. These were measured as described by Dalgleish & Peacocke (1971) with a Jouan Dichrographe. The solutions used were of β -lactamase I (about 1.5 mg/ml) in Tris- Zn^{2+} buffer or in this buffer containing different concentrations of urea. The spectra of heated solutions were measured in the 0.5 mm cuvettes of the instrument at given temperatures after they had been maintained at these temperatures for 20 min and, in some cases, also after 10 min. A single solution was used for all measurements in the same series, the temperature being raised stepwise after the first spectrum had been determined.

The c.d. spectra are expressed in units of $10^2 \times \Delta E$, where ΔE is the measured chart reading for a given concentration of protein and is proportional to $\Delta \epsilon$ ($\epsilon_e - \epsilon_r$), the difference in molar extinction coefficients for left- and right-circularly polarized light.

Effect of denaturing agents on c.d. spectra and activity. For these experiments a solution of β -lactamase I (1.5 mg/ml) in Tris- Zn^{2+} buffer was dialysed for 18 h at 4°C against 100 vol. of a solution (two changes) containing different concentrations of urea, or guanidinium chloride in Tris- Zn^{2+} buffer. After

* Present address: The Hannah Research Institute, Ayr KA6 5HL, U.K.

dialysis the volume of the protein solution was re-adjusted to its original value by addition of diffusate. For β -lactamase assays samples of the resulting solutions (1 or 2 μ l) were pipetted into a solution (2ml) containing benzylpenicillin (1.5mg/ml) in the corresponding concentration of denaturing agent.

Changes in activity with pH. Samples of a solution of β -lactamase I in Tris- Zn^{2+} buffer (about 1.5mg/ml) were adjusted to pH 3.0 with 12M-HCl or to pH 11.0 with 10M-NaOH. For measurements of β -lactamase activity small samples (1 or 2 μ l) of these solutions were pipetted at intervals into the assay solution (2.0ml) containing benzylpenicillin as substrate and the pH was maintained at 7.0.

Results

Reversible denaturation by heat

When a solution of β -lactamase I (1.5mg/ml) in Tris- Zn^{2+} buffer, pH 7.0, was kept at 60°C for 30 min a voluminous white precipitate appeared. After removal of the precipitate by centrifugation the supernatant solution showed an absorbance at 280nm which was very small ($E=0.05$) in comparison with that of the original solution ($E=1.6$).

The washed precipitate, which showed no detectable activity when suspended in 0.5M-NaCl, dissolved readily in 8M-urea or 5M-guanidinium chloride.

Dialysis of the resulting solutions against 100vol. of Tris- Zn^{2+} buffer (two changes), or gel filtration through Sephadex G-25 (coarse grade) in the same buffer, resulted in almost complete (up to 98%) recovery of activity. In some experiments a small amount of white inactive material (5–10% of the total) remained suspended in the solution after dialysis. The latter redissolved in 8M-urea or 5M-guanidinium chloride, but re-formed on dialysis of the solution.

The circular-dichroism spectra and the change in ΔE at 220nm of a solution of β -lactamase I which had been maintained for successive periods of 20 min at different temperatures are shown in Figs. 1(a) and 1(b) respectively. The spectra measured after only 10 min were not significantly different, indicating that relatively stable states had been reached. A large change in ΔE occurred between 50° and 55°C. Between 55° and 65°C the shapes of the spectra were similar and the decrease in ΔE in this range may be attributed to loss of material from solution by precipitation, or to effects arising from solution turbidity, rather than to a further change in conformation. Precipitation became apparent at 60°C and was considerable at 65°C.

The relative activities of solutions of β -lactamase I which had been kept for 5 and 15 min respectively at temperatures from 30° to 60°C and then assayed

Fig. 1. Effect of changes in temperature on the circular-dichroism spectra and activity of β -lactamase I

(a) Spectra of β -lactamase I after heating for 20 min at: (1) 30°C; (2) 40°C; (3) 50°C; (4) 55°C; (5) 60°C; (6) 65°C. (b) Changes in ΔE at 220nm and in β -lactamase activity. ●, ΔE ; ○, activity after 5 min; ▲, activity after 15 min (relative to an arbitrary value of 100 at 30°C). For details see the text.

immediately at the same temperature are also shown in Fig. 1(b). Between 30° and 50°C the activity increased with temperature and was virtually the same after 15 min as after 5 min incubation. Rapid inactivation began between 50° and 55°C and was complete within 5 min at 60°C.

Changes in the presence of denaturing agents

The circular-dichroism spectra of solutions of β -lactamase I which had been dialysed against different concentrations of urea and the variation in ΔE at 220 nm with urea concentration are shown in Figs. 2(a) and 2(b) respectively. The shape of the spectrum of the denatured protein in 6M-urea is different from that of the protein denatured by heat (Fig. 1).

Fig. 2(b) also shows the change observed in the activity of β -lactamase I when the latter was dialysed against (and then assayed in) solutions containing different concentrations of urea (1–8M). The activity fell with increasing concentrations of urea until it was virtually zero in a urea concentration of about 5M. The loss of activity in 8M-urea (or in 5M-guanidinium chloride) was rapidly reversible. When a 1 or 2 μ l sample of a solution of β -lactamase I in either of these denaturing agents was pipetted into the routine assay solution (2.0ml) the rate of hydrolysis of the

benzylpenicillin substrate increased during the first 3 min. The final rate approached that observed with a corresponding sample of enzyme which had not been treated with denaturing agent.

The circular-dichroism spectra of β -lactamase I which had been dialysed against 2M-urea and then kept for successive periods of 20 min at different temperatures are shown in Fig. 3(a) and the change in ΔE at 220 nm with temperature is shown in Fig. 3(b). No change appeared to have occurred in these spectra between 10 and 20 min. As expected, the presence of urea decreases the temperature at which rapid denaturation occurs. The circular-dichroism spectrum of the denatured form is different in shape from those obtained after denaturation at 60°C in Tris- Zn^{2+} buffer, or in 6M-urea at room temperature, and the decrease in ΔE is smaller.

Effects of changes in pH

When a solution of β -lactamase I was kept at room temperature for 30 min at pH 3.0 and a small sample (1 or 2 μ l) then pipetted into the assay solution (2.0ml) at pH 7.0 the initial rate of hydrolysis of substrate was about 15% of that observed with a corresponding solution of enzyme maintained at pH 7.0. The initial rate of hydrolysis by enzyme which

Fig. 2. Effect of urea on the circular-dichroism spectra of β -lactamase I

(a) Spectra of β -lactamase I in: (1) Tris- Zn^{2+} buffer, pH 7.0; (2), (3) and (4) buffer containing 2, 4 and 6M-urea respectively. (b) Change in activity and ΔE at 220 nm with urea concentration. For details see the text. $\circ$, β -Lactamase activity (relative to an arbitrary value of 100 for the activity in buffer alone); $\bullet$, ΔE .

Fig. 3. Effect of changes in temperature on the circular-dichroism spectra of β -lactamase I in the presence of urea

(a) Spectra of a solution of β -lactamase I kept for 20 min periods at successively higher temperatures in 2M-urea. (1) 30°C; (2) 40°C; (3) 45°C. (b) Change in ΔE with temperature at 220 nm.

Fig. 4. Rate of hydrolysis of benzylpenicillin at pH 7.0 by a solution of β -lactamase I which had been kept at room temperature for 45 min after adjustment of the pH to 11.0

The arrow shows the time of addition of enzyme to substrate. For details see the text.

Fig. 5. Change in activity with time at room temperature of solutions of β -lactamase I whose pH had been adjusted to (a) 3.0 and (b) 11.0

●, Initial rate of hydrolysis on readjustment of pH to 7.0; ○, rate after 3 min; activities are relative to an arbitrary value of 100 for the activity of a control solution at pH 7.0. For details see the text.

had been kept for 30 min after adjustment of the pH to 11.0 was about 45% of the original value. In both cases, however, the rate of hydrolysis increased during the first 4 min of the assay before showing zero-order kinetics (Fig. 4). Fig. 5 shows the initial and final rates of hydrolysis of benzylpenicillin at pH 7.0 by portions of the same solution of β -lactamase I which had been kept for various times at pH 3.0 and 11.0 respectively. In both cases the loss of enzymic activity as estimated from initial rates of hydrolysis increased progressively with time and at pH 3.0 appeared to

occur in two phases. The proportion of lost activity which was regained at pH 7.0 also increased with time, but virtually all that lost between 15 and 45 min at pH 11.0 was recovered.

Discussion

A sudden and extensive loss in the activity of β -lactamase I occurred when the temperature of a solution of the enzyme at pH 7.0 was raised above 50°C.

Between 50° and 55°C the activity fell to less than 10% of its original value within 5 min and at 60°C to an undetectable value. Circular-dichroism spectra showed that the rapid loss of activity between 50° and 55°C was accompanied by an extensive conformational change. The spectrum of the high-temperature conformation did not appear similar to those known for ordered structures, but it did not correspond to that typical of unordered proteins such as histones (Dalglish, 1972), and it is probable that complete disordering of the molecule had not occurred. Precipitation of the inactivated enzyme from solution appeared to follow the major change in conformation, but the recovery of a soluble enzyme with almost all the original activity after solution of the precipitate in a denaturing agent and removal of the latter indicated that both changes were reversible.

In agreement with the findings of Imsande *et al.* (1970) with penicillinase from *B. cereus* 569, the denaturation and complete loss of activity of β -lactamase I in 5M-guanidinium chloride or 8M-urea was fully reversible. The kinetics of hydrolysis of substrate, observed when the denaturing agent was suddenly diluted in the assay solution at 30°C, showed that refolding of the molecule occurred rapidly and was complete within 5 min. The circular-dichroism spectrum of the protein changed progressively as the urea concentration was raised from 2 to 5M. The spectrum of the denatured form in 5M-urea was different from that of the form produced by heat-treatment and was more representative of the typical circular-dichroism spectra of proteins treated with urea and supposedly an unordered form. Both enzymic activity and circular-dichroism spectra show that even at low urea concentrations (2M) some change in protein conformation occurs. This may involve an equilibrium between native and partly unfolded proteins. However, the difference in the shape of the curves obtained by plotting urea concentration against enzymic activity and ΔE at 220 nm respectively (Fig. 2b) indicates that in solutions containing 2–4M-urea the protein is not present merely in two states, one native and one unordered, in equilibrium.

In 2M-urea β -lactamase I underwent a rapid change

in conformation between 35° and 45°C. The new conformation (or mixture of conformations) was different from that produced at 60°C in the absence of a denaturing agent and also from that assumed at room temperature in 6M-urea. The smaller magnitude of the change in extinction suggests that it may differ less than either of the latter from the conformation of the original enzyme.

Some, at least, of the loss of activity of β -lactamase I at pH 3.0 and 11.0 is due to conformational changes because it is readily reversible, refolding apparently occurring at pH 7.0 within 5 min. The shapes of the activity curves reflected by the changes at pH 11.0 might be accounted for if the enzyme first assumed a new and relatively stable conformation of lower activity and if the latter underwent further changes that were reversible at pH 7.0. Further studies are required to relate these findings to those on the conversion of β -lactamase I (α -penicillinase) in dilute alkali into the iodine-sensitive γ state (Citri, 1958), in which a tyrosine residue in the molecule becomes exposed (Czanyi *et al.*, 1971).

We are grateful to the Medical Research Council and the National Research Development Corporation for financial support and to Dr. S. G. Waley for helpful discussions.

References

- Citri, N. (1958) *Biochim. Biophys. Acta* **27**, 277–281
- Citri, N., Garber, N. & Sela, M. (1960) *J. Biol. Chem.* **235**, 3454–3459
- Crompton, B., Jago, M., Crawford, K., Newton, G. G. F. & Abraham, E. P. (1962) *Biochem. J.* **83**, 52–63
- Czanyi, V., Ferencz, I. & Mile, I. (1971) *Biochim. Biophys. Acta* **236**, 619–627
- Dalglish, D. G. (1972) *FEBS Lett.* **24**, 134–136
- Dalglish, D. G. & Peacocke, A. R. (1971) *Biochem. J.* **125**, 155–158
- Davies, R. B., Abraham, E. P. & Melling, J. (1974) *Biochem. J.* **143**, 115–127
- Imsande, J., Gillin, F. D., Tanis, R. J. & Atherley, A. G. (1970) *J. Biol. Chem.* **245**, 2205–2212
- Kuwabara, S. (1970) *Biochem. J.* **118**, 457–465
- Rudzik, M. B. & Imsande, J. (1970) *J. Biol. Chem.* **245**, 3556–3564