Metal Cofactor Requirements of β -Lactamase II

By RICHARD B. DAVIES and E. P. ABRAHAM

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

(Received 30 May 1974)

1. The apoenzyme obtained on removal of Zn^{2+} from β -lactamase II from *Bacillus cereus* 569/H/9 showed less than 0.001% of the activity of the Zn^{2+} -containing enzyme.
2. Removal of Zn^{2+} led to a conformational change in the enzyme and partial unmasking of a thiol group.
3. Replacement of Zn^{2+} by Co^{2+} , Cd^{2+} , Mn^{2+} or Hg^{2+} gave enzymes with significant, but lower, β -lactamase activity. No activity was detected in the presence of Cu^{2+} , Ni^{2+} , Mg^{2+} or Ca^{2+} .
4. Equilibrium dialysis indicated that the enzyme had at least two Zn^{2+} binding sites. With benzylpenicillin as substrate the variation in activity with concentration of Zn^{2+} indicated that activity paralleled binding of Zn^{2+} to the site of highest affinity.
5. Replacement of Zn^{2+} by Co^{2+} and Cd^{2+} gave enzymes with absorption bands at 340 and 245 nm respectively, and raised the question of whether the thiol group in the enzyme is a metal-ion ligand.
6. Reduction of the product obtained by reaction of denatured β -lactamase II with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] gave a protein which could refold to produce β -lactamase II activity in high yield.

β -Lactamase II from *Bacillus cereus* 569/H differs from β -lactamase I in that Zn^{2+} is a cofactor (Sabath & Abraham, 1966) and in containing a cysteine residue (Kuwabara *et al.*, 1970; Davies *et al.*, 1974), as well as in other properties. Sabath & Finland (1968) found that crude Zn^{2+} -deficient cephalosporinase (β -lactamase II) from *B. cereus* 569 was no longer activated by addition of Zn^{2+} after treatment with iodoacetate, *N*-ethylmaleimide or *p*-chloromercuribenzoate, and suggested that a thiol group in the enzyme is a Zn^{2+} ligand. Further experiments relating to the metal requirements of the enzyme and the role of the thiol group are described in the present paper.

Materials and Methods

Materials

Reagents. Carrier-free $^{65}\text{ZnCl}_2$ in 0.1M-HCl (188 Ci/g; 1 $\mu\text{g/ml}$) and [$1\text{-}^{14}\text{C}$]phenylacetic acid (0.1mCi; 0.6mg) were from The Radiochemical Centre, Amersham, Bucks., U.K. Ellman's (1959) reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] was from Ralph N. Emanuel, Wembley, Middx., U.K. Cleland's reagent (dithiothreitol) was from Calbiochem, London W.1, U.K. Penicillin and cephalosporin substrates, and 'Tris', 'Tris- Zn^{2+} ', 'Tris-salt' and 'Tris- Zn^{2+} -salt' buffers were as described by Davies *et al.* (1974). For the preparation of Zn^{2+} -free buffers used in the determination of Zn^{2+} , the buffers were shaken with 0.07% (w/v) dithizone in carbon tetrachloride until the organic phase remained

green and then with carbon tetrachloride alone (Malmström, 1956), or were treated with Chelex resin (100–200 mesh; Na^+ form) encased in Visking dialysis tubing.

β -Lactamases. The preparations of β -lactamase I and β -lactamase II used were purified by the method of Davies *et al.* (1974).

General methods

Assays of β -lactamase activities, determinations of protein, and electrophoresis and chromatography on paper were carried out as described by Davies *et al.* (1974).

Measurements of radioactivity. The radioactivities of solutions containing $^{65}\text{Zn}^{2+}$ were determined with a Nuclear-Chicago Unilux IIA scintillation counter in the water-miscible scintillation fluid of Bruno & Christian (1960). The counting efficiency was about 10%.

Ultraviolet-visible absorption spectra. For measurements of the absorption spectra of β -lactamase II in the presence of different metal ions a solution of the enzyme in Tris- Zn^{2+} buffer (8ml; 6.5mg/ml) was diluted to 10ml with EDTA (50mM; pH 7.0) and applied at 0°C to a column (84cm $\times$ 2.5cm diam.) of Sephadex G-25 (medium grade) prepared in Tris buffer which had been freed from traces of Zn^{2+} . Elution was carried out with Zn^{2+} -free Tris-salt buffer and 30 fractions (each 10ml) were collected. The fraction containing the highest concentration of Zn^{2+} -free β -lactamase II was diluted with eluent to give a solution with an extinction of 2.39 at 280nm

(equivalent to an enzyme concentration of approx. 0.1mM). To samples of this solution (1ml) in the spectrophotometer cell were added appropriate amounts (generally 1 μ l) of 1M-CoSO₄·7H₂O, 1M-CdSO₄·7H₂O or 1M-ZnSO₄·7H₂O in Tris buffer. Similar additions were made to solutions of β -lactamase II in 1mM-ZnSO₄. Absorption spectra between 300 and 700nm were recorded with an SP.800 spectrophotometer (Pye Unicam, Cambridge, Cambs., U.K.) in conjunction with a Servoscribe recorder (type RE 511; Smiths Industries Ltd., Cricklewood, London NW2 7UR, U.K.) maximum energy settings and the expanded scale being used. Difference spectra were measured by the four-cell procedure.

The Zn²⁺ content of Zn²⁺-free β -lactamase II was determined by the method of Malstrom (1956). In two preparations there was less than 0.005g-atom of Zn²⁺/mol of protein and in a third 0.044g-atom of Zn²⁺/mol of protein.

Circular-dichroism spectra. These were measured in the manner described by Dalglish & Peacocke (1971), the protein solutions used (about 1.5mg/ml) being β -lactamase I and β -lactamase II respectively in Tris-Zn²⁺ buffer, and Zn²⁺-free β -lactamase II (prepared as for measurements of absorption spectra) in Tris-salt buffer.

Binding of Zn²⁺ to β -lactamase II. This was studied by equilibrium dialysis in a cell similar to that of Myer & Schellman (1962). The cell was soaked in 50mM-EDTA and then washed well with water. The membrane dividing the two halves of the cell was cut from Visking dialysis tubing (Union Carbide Corp., Chicago, Ill., U.S.A.) and treated with 50% (v/v) ethanol and with EDTA as described by McPhie (1971). In the absence of protein no uptake of ⁶⁵Zn²⁺ by the cell or the membrane was detected. A Ballotini bead (40mg) was inserted into each half of the cell. The latter was rotated at room temperature in an elliptical path (axes 4cm and 6cm) at 90rev./min. A solution (2.5ml) of Zn²⁺-free β -lactamase II (0.035mM) in Tris-salt buffer (prepared as for measurements of absorption spectra) was placed on one side of the membrane and ⁶⁵ZnSO₄ (2.5ml; 1mM–10nM) in Tris-salt buffer on the other. The latter solutions were made by addition of 10 μ l of carrier-free ⁶⁵ZnCl₂ solution (1.88 μ Ci) to solutions containing different concentrations of ZnSO₄. After 20h, when equilibrium had been reached, samples (0.1 or 0.2ml) were removed from each compartment for determinations of radioactivity.

Stability constants of complexes of Zn²⁺ with cephalosporin C and Tris. These constants were determined by the potentiometric method of Albert (1950). Titrations were carried out at 30°C in an automatic recording titrator (Radiometer, Copenhagen, Denmark).

A solution of cephalosporin C in 0.5M-NaCl was adjusted to pH4.0 with 1M-HCl and diluted with 0.5M-NaCl to give a solution (5ml; 0.01M) which was titrated to pH10.0 with 0.2M-NaOH. Blank corrections were obtained from a titration in the absence of cephalosporin C. The pK_a value obtained for the free amino group of cephalosporin C was 9.29 [cf. the value of 9.8 obtained by Newton & Abraham (1956) at room temperature]. Further titration curves were obtained similarly in the presence of 0.01M-ZnSO₄, but titration was stopped at pH7.5 since precipitation occurred at this point.

Tris (0.01M) in 0.5M-NaCl was titrated from pH4.0 in the presence and in the absence of 0.01M-ZnSO₄ in the manner described for the titration of cephalosporin C. The pK_a of Tris at 30°C was 8.0 [cf. the value of 8.3 at 20°C given by Good *et al.* (1966)]. From a limited number of values obtained by titration between pH5.0 and 6.6 in the presence of ZnSO₄ the absolute stability constant was calculated to be between 0.5 and 2.0litre/mol. Conversion into an apparent stability constant at pH7.0 (Sullivan, 1969) gave values between 0.05 and 0.2litre/mol. The binding of Zn²⁺ to the Tris buffer was therefore taken to be negligible in subsequent experiments.

Reaction with Ellman's reagent. (a) A solution of β -lactamase II (about 8mg/ml) in Tris-Zn²⁺ buffer was adjusted to pH8.0 and the following solutions (total vol. 1.2ml) were then made up in spectrophotometer cells (path-length 10mm): (i) β -lactamase II (0.3ml), water (0.9ml); (ii) β -lactamase II (0.3ml), water (0.6ml), EDTA (50mM; 0.3ml); (iii) β -lactamase II (0.3ml), water (0.15ml), EDTA (50mM; 0.3ml), guanidinium chloride (0.6g). Ellman's reagent was dissolved in potassium phosphate buffer (0.1M in K⁺; pH7.0) to give a concentration of 0.01M. Samples (7 μ l) of this solution were added with mixing to the solutions in the cells and the increase in extinction at 412nm was recorded. A control experiment showed that ZnSO₄ in the concentration used did not affect the change in extinction at 412nm when Ellman's reagent reacted with *N*-acetylcysteine (0.08mM) in the potassium phosphate buffer at pH8.0.

(b) A solution of β -lactamase II (8ml; 2.5mg/ml) in equal volumes of 50mM-EDTA and Tris buffer containing 5M-guanidinium chloride was adjusted to pH8.0 with 1M-NaOH. A fivefold excess of Ellman's reagent (0.01M) in the potassium phosphate buffer (0.5ml) was added and the mixture left at 30°C overnight. The protein product of the reaction was isolated by gel filtration on a column (84cm $\times$ 2.5cm diam.) of Sephadex G-25 (medium grade) at 0°C, the eluent being Zn²⁺-free Tris-salt buffer. The u.v.-absorption spectrum of the protein fraction of the eluate showed a band with λ_{max} . 324nm, as well as one with λ_{max} . 280nm, E_{280}/E_{324} being 1/0.212.

Results

Effect of different metal ions on the activity of β -lactamase II

After β -lactamase II in Tris- Zn^{2+} buffer had been dialysed overnight at 4°C against 10 vol. of 50 mM-EDTA (adjusted to pH 7.0 with 12 M-HCl) the solution showed less than 0.001% of its original activity. When small samples (1–2 μ l) of this solution were added to the routine assay solution (2.0 ml) containing 0.35 mM- ZnSO_4 (Davies *et al.*, 1974) virtually all the activity was restored. When similar samples were added to a solution in which the ZnSO_4 had been replaced by the sulphates of Co^{2+} , Cd^{2+} or Mn^{2+} , or by HgCl_2 , in the same molecular concentrations, the β -lactamase activity was 10.7, 10.5, 2.9 and 4.4% respectively of that in the presence of Zn^{2+} . When Zn^{2+} was replaced by the sulphates of Cu^{2+} , Ni^{2+} or Mg^{2+} , or by CaCl_2 , no activity was detected.

In the presence of 0.35 mM- HgCl_2 the rate of hydrolysis (initially 4.4% of that with 0.35 mM- ZnSO_4) decreased rapidly during 10 min and fell to zero when only 50% of the cephalosporin C substrate had been hydrolysed. By this time a yellow precipitate had formed in the solution. Addition of an equimolar amount of ZnSO_4 did not result in renewed hydrolysis.

Fig. 1. Binding of Zn^{2+} to β -lactamase II

The values for Zn^{2+} bound (●) are median values for from two to seven determinations. Estimations of the precision of the highest and lowest values for Zn^{2+} binding gave values of $\pm 20\%$ and $\pm 22\%$ respectively with 90% confidence limits. Calculations were made as described by Dean & Dixon (1951) from seven determinations of diffusible Zn^{2+} and of diffusible Zn^{2+} plus protein-bound Zn^{2+} at equilibrium.

Binding of Zn^{2+} to β -lactamase II

Fig. 1 shows the values obtained for g-atoms of Zn^{2+} bound/mol of protein at different values of $\log[\text{Zn}^{2+}]$, the protein used being Zn^{2+} -free β -lactamase II and the concentration of Zn^{2+} being that of the total diffusible Zn^{2+} in the equilibrium dialysis cell. The results indicated that there were at least two binding sites with widely different affinity constants. About 0.8 g-atom of Zn^{2+} was bound/mol of protein at a concentration of Zn^{2+} of 10 μ M. If this binding occurred at a single immobile site the amount of Zn^{2+} bound should fall from 0.8 g-atom to 0.2 g-atom/mol of protein, with a decrease of 1.2 units in $\log[\text{free } \text{Zn}^{2+}]$. The fall shown in Fig. 1, from 0.8 g-atom at a value for Zn^{2+} of 10 μ M to almost zero at a value of 1 μ M, is thus more rapid than that expected on this basis.

Variation in rates of hydrolysis with ZnSO_4 and CoSO_4 concentrations

(a) The rate of hydrolysis of benzylpenicillin (4 mM) appeared to approach a maximum when an amount of ZnSO_4 corresponding to a final concentration of 1 mM, or CoSO_4 to a concentration of 10 mM, was added to Zn^{2+} -free β -lactamase II prepared by treatment of the Zn^{2+} enzyme with EDTA and chromatography on Sephadex G-25. However, the rate of hydrolysis in the presence of 10 mM- CoSO_4 was only about 20% of that in the presence of 1 mM- ZnSO_4 .

Fig. 2 shows plots of $\log[\text{ZnSO}_4]$ and $\log[\text{CoSO}_4]$ against β -lactamase activity with benzylpenicillin (4 mM) as substrate. In the assay system the concentration of enzyme is too low (approx. 1 nM) to decrease significantly the concentration of free Zn^{2+} in the solution ($\geq 1 \mu$ M). Hence the fall in relative activity shown in Fig. 2 from 80% of its maximum at about 10 μ M- ZnSO_4 to almost zero at 1 μ M- ZnSO_4 resembles the fall in the amount of Zn^{2+} bound shown in Fig. 1 and is also more rapid than that calculated for a single immobile binding site.

(b) A plot of $\log[\text{ZnSO}_4]$ against relative β -lactamase II activity with cephalosporin C as substrate appeared to give a biphasic curve. In this case the question arose whether the rate of hydrolysis was affected by the known ability of cephalosporin C to form a Zn^{2+} complex (Bickel *et al.*, 1969). The cumulative stability constant of the cephalosporin C complex (K_1K_2) was found to be $10^{8.4}$ litre/mol and the first stability constant (K_1) $10^{5.0}$ litre/mol. The apparent stability constants at pH 7.0 were calculated to be $10^{2.7}$ litre/mol and $10^{-1.2}$ litre/mol. The concentration of cephalosporin C in the assay system was 3 mM. From the first apparent stability constant of $10^{2.7}$ the decreases in the concentration of Zn^{2+} by formation of a cephalosporin C- Zn^{2+} complex

Fig. 2. Initial rates of hydrolysis of benzylpenicillin (4 mM) by β -lactamase II in different concentrations (M) of $ZnSO_4$ (○) and $CoSO_4$ (▲)

The concentrations correspond to the total amounts of $ZnSO_4$ and $CoSO_4$ (M^{2+}) respectively added to the Zn^{2+} -free enzyme. The precision of the values for rates of hydrolysis (relative to an arbitrary value of 100 with 1 mM- $ZnSO_4$), as estimated by the procedure of Dean & Dixon (1951) from six successive assays under standard conditions of samples of the same enzyme solution, was $\pm 2.8\%$ with 95% confidence limits.

Fig. 3. Relationship of Zn^{2+} binding of β -lactamase II to β -lactamase activity with cephalosporin C as substrate

●, Zn^{2+} binding; ○, relative activity. In the determinations of enzymic activity the values given for free Zn^{2+} are those for total Zn^{2+} minus those calculated for Zn^{2+} chelated with the substrate. An arbitrary value of 100 has been assigned to the activity at the highest concentration of Zn^{2+} used.

were calculated for different quantities of added $ZnSO_4$. Thus when the total $ZnSO_4$ concentrations were 1 μM and 10 mM the concentrations of free Zn^{2+} , calculated on this basis, were 0.4 μM and 7.6 mM respectively. Fig. 3 shows a plot of the calculated $\log[\text{free } Zn^{2+}]$ against activity together with the values for Zn^{2+} binding determined by equilibrium dialysis. The shape of the curve indicated that maximum activity was associated with binding of Zn^{2+} at more than one site, but the first and rapid rise in activity occurred at a significantly lower apparent concentration of free Zn^{2+} than did the corresponding rise in the amount of Zn^{2+} bound to protein.

Thiol group of β -lactamase II as a possible metal ligand

Fig. 4 shows a difference spectrum of Zn^{2+} -free β -lactamase II (0.1 mM) in Tris buffer containing 5 mM- $CoSO_4$ and in Tris buffer alone. Addition of Co^{2+} resulted in the appearance of an absorption band with λ_{max} , 340 nm. The value of E_{340} increased by 90% as the concentration of Co^{2+} was raised from 1 to 5 mM, but at concentrations of 10 mM or more, precipitation of the protein occurred.

A difference spectrum was also measured for β -lactamase II (0.1 mM) in Tris- Zn^{2+} buffer (1 mM with respect to $ZnSO_4$) and the same solution to which $CdSO_4$ had been added. On addition of Cd^{2+} (final concn. 1 mM) an absorption band appeared with λ_{max} , 245 nm. The extinction at this wavelength

Fig. 4. Difference spectrum at pH 7.0 in Tris buffer of Zn^{2+} -free β -lactamase II (0.1 mM) in the presence and in the absence of 5 mM- $CoSO_4$

The wavelength scale (nm/cm) below 350 nm is half that at longer wavelengths.

increased rapidly with time and reached a maximum ($E_{1\text{cm}} = 0.05$) within 8 min. A further increase in extinction to 0.07 occurred after the addition of a second equal amount of CdSO_4 .

Fig. 5. Reaction of β -lactamase II with Ellman's reagent

Curve (A), in Tris- Zn^{2+} buffer; curve (B), after addition of EDTA (final concn. 12.5 mM) to buffer for curve (A); curve (C), after addition of guanidinium chloride (final concn. 5 M) to buffer for curve (B). Curve (D) is a continuation of curve (B) after 18 h. See the text for details.

β -Lactamase II did not react significantly with Ellman's reagent in Tris- Zn^{2+} buffer (Fig. 5). In the same buffer, but in the presence of excess of EDTA, a slow increase in extinction at 412 nm occurred and corresponded, after 18 h, to the reaction of 1.0 thiol group/molecule of protein. When the reaction mixture contained, in addition, 5 M-guanidinium chloride, the reaction was complete within 4 min.

The circular-dichroism spectra of β -lactamase II and the Zn^{2+} -free protein are shown in Fig. 6. Removal of Zn^{2+} led to a significant change in the spectrum, both in the near u.v. (indicative of a change in the environment of the aromatic residues) and in the far u.v. (indicative of a change in the folding of the peptide chain).

Zn^{2+} -free β -lactamase II retained most, at least, of the thermostability of the Zn^{2+} -containing enzyme. Thus on being kept at 60°C for 30 min, a solution of β -lactamase II in Tris- Zn^{2+} buffer showed virtually no change in activity and the activity of a similar solution containing 50 mM-EDTA was restored to 72% of its original value in the presence of excess of Zn^{2+} .

Reversible denaturation of β -lactamase II

The product obtained by reaction of β -lactamase II in 5 M-guanidinium chloride with Ellman's reagent showed no Zn^{2+} binding comparable with that of the original protein after removal of the denaturing

Fig. 6. Circular-dichroism spectra of β -lactamase II in Tris- Zn^{2+} buffer and of Zn^{2+} -free β -lactamase II in Tris buffer

----, β -Lactamase II; —, apoenzyme.

agent. It showed 2.5% of the activity of the original enzyme when diluted in the routine assay solution, but this activity may have been due to the renaturation of a small amount of enzyme which had not formed a mixed disulphide. Incubation of the solution of the product with 0.1% dithiothreitol overnight, followed by gel filtration through a column of Sephadex G-25 with Tris- Zn^{2+} buffer as eluent, gave a protein fraction with 77% of the activity of the original preparation.

Discussion

Sabath & Finland (1968) concluded that a number of metal ions would compete with Zn^{2+} for reaction with a thiol group in the enzyme responsible for the cephalosporinase activity of culture filtrates of *B. cereus* 569, since HgCl_2 and CdCl_2 prevented activation of the crude enzyme by Zn^{2+} and NiCl_2 partially inhibited the ability of *N*-ethylmaleimide to prevent Zn^{2+} activation. They suggested that the relative affinities of these metal ions for the thiol group decreased in the series $\text{Hg}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+}$. However, before the present work Zn^{2+} was the only metal ion known to activate Zn^{2+} -free β -lactamase II.

The experiments described here show that Co^{2+} , Cd^{2+} , Mn^{2+} and Hg^{2+} form metal-enzyme complexes which have significant β -lactamase activity, although much lower than that of the Zn^{2+} enzyme. The finding that Co^{2+} was one of the most effective substitutes for Zn^{2+} among the metal ions tested is in accordance with the close resemblance of these two ions and the known ability of Co^{2+} to replace Zn^{2+} in a number of other metallo-enzymes (Vallee & Williams, 1968; Foster *et al.*, 1970).

Although the data obtained from equilibrium dialysis and measurements of rates of hydrolysis were limited, they indicated that β -lactamase II could bind more than one g-atom of Zn^{2+} /mol and that its activity with benzylpenicillin as a substrate was a function of the Zn^{2+} bound by the site of highest affinity. However, the maximum rate of increase found in both the bound Zn^{2+} and in β -lactamase activity with the estimated $\log[\text{free Zn}^{2+}]$ was greater than that calculated for a single immobile binding site in the enzyme. The second Zn^{2+} -binding site appeared to have too low a stability constant, relative to that of the first, for it to exert a positive co-operative effect (Fig. 2). Weber & Anderson (1965) have predicted that a single protein site will appear to show positive co-operative binding if the protein-ligand complex undergoes a conformational change that increases its stability constant, provided that the time required for this change is greater than the average lifetime of the complex, or that the time of relaxation of the protein to its original conforma-

tion is greater than the average lifetime of the uncomplexed protein, or both. At a low ligand concentration the lower stability constant will then be involved, but the higher constant will begin to operate as the ligand concentration increases. In the present case, however, other explanations of the findings have not been eliminated. The binding of very small amounts of Zn^{2+} to glass in the β -lactamase assay system would not have been detected. The Zn^{2+} -free enzyme was prepared by removal of the Zn^{2+} with EDTA. In view of the high stability constant ($10^{16.6}$ litre/mol) of the Zn^{2+} -EDTA complex (Sullivan, 1969), contamination of the apoenzyme with EDTA could lower the concentration of free Zn^{2+} to a very low value until after an amount of Zn^{2+} equivalent to the EDTA had been added. The estimated value for $\log[\text{free Zn}^{2+}]$ would then be significantly greater than the true value at the beginning, but not at the end, of the Zn^{2+} -binding curve.

With cephalosporin C as a substrate the apparent increase in β -lactamase activity with $\log[\text{free Zn}^{2+}]$ after the latter had reached a value corresponding to the binding of one g-atom of Zn^{2+} /mol of protein (Fig. 3) could possibly be associated with the formation of a cephalosporin C- Zn^{2+} complex which provided a more readily hydrolysable substrate. Binding of Zn^{2+} to cephalosporin C [$K_1(\text{app.}) = 10^{2.7}$ litre/mol] would occur within the range of binding of a second Zn^{2+} to the protein.

If only the enzyme- Zn^{2+} complex (and not the apoenzyme) were able to combine with substrate the curve obtained from a plot of β -lactamase activity against $\log[\text{free Zn}^{2+}]$ would be displaced from the corresponding Zn^{2+} -binding curve towards lower concentrations of free Zn^{2+} . Combination with substrate would affect the equilibrium between the apoenzyme, free Zn^{2+} and the enzyme-metal complex to an extent depending on the dissociation constant of the complex and the concentration of substrate. A displacement was observed with cephalosporin C as substrate (Fig. 3), but not with benzylpenicillin (Figs. 1 and 2). However, more extensive studies of Zn^{2+} binding and of its effect on β -lactamase activity are required to throw light on the reaction mechanism and to obtain reliable values for the affinity constants involved.

The finding that Zn^{2+} -free β -lactamase II reacted with Ellman's reagent, whereas its Zn^{2+} complex did not, and that the product of the reaction with Ellman's reagent could be reconverted into the active enzyme by reduction with dithiothreitol and subsequent addition of Zn^{2+} , is consistent with the results of Sabath & Finland (1968) with less specific thiol-group reagents. It is clear that the thiol group is partly unmasked on removal of Zn^{2+} and that added Zn^{2+} does not reactivate the enzyme after reaction of the thiol group with reagents so far used.

In showing absorption bands with λ_{max} 340 and

245nm, the absorption spectra of β -lactamase II with bound Co^{2+} and Cd^{2+} respectively resemble those found by Drum & Vallee (1970) for cobalt-containing horse liver alcohol dehydrogenase (λ_{max} . 340nm) and the corresponding cadmium-containing enzyme (λ_{max} . 245nm). A charge-transfer band at 375nm is shown by cobalt(II)-cysteine complexes (Foster *et al.*, 1970). A band near 250nm is found in native metallothionein (which contains both Zn^{2+} and Cd^{2+}) and is analogous to that of cadmium mercaptide complexes (Foster *et al.*, 1970; Vallee & Wacker, 1970).

The absorption spectra of cobalt- and cadmium-containing β -lactamase II appear to be consistent with the suggestion that the thiol group in the enzyme acts as a metal ligand. They do not yet prove that this is so, nor, if it is, do they indicate whether the thiol group is involved in metal binding at the site of highest affinity. On the basis of the concentration of protein, the values for the apparent molar extinctions of β -lactamase II in the presence of Co^{2+} (λ_{max} . 340nm) and Cd^{2+} (λ_{max} . 245nm) were only 10 and 7% respectively of those reported for cobalt- and cadmium-containing liver alcohol dehydrogenase containing 3.8g-atoms of metal/mol (Drum & Vallee, 1970). The relationships of the extinctions at these wavelengths to the amounts of metal bound by the β -lactamase remain to be determined.

The present findings show that the thiol group and features of a Zn^{2+} -binding site are mutually affected by changes in each other. Further data are required to establish whether Zn^{2+} is bound directly to sulphur, or whether the binding of Zn^{2+} to other ligands stabilizes a protein conformation in which the thiol group is inaccessible. The occurrence of a conformational change in the protein on removal of Zn^{2+} is revealed by a change in circular dichroism.

We are grateful to the Medical Research Council and the National Research Development Corporation for financial support. We are indebted to Dr. D. G. Dalgleish for measurements of circular-dichroism spectra and to Dr. S. G. Waley for helpful discussions.

References

- Albert, A. (1950) *Biochem. J.* **47**, 531–538
Bickel, H., Bosshardt, R., Mueller, J., Fechtig, B., Voser, W. & Peter, H. (1969) *Can. Patent* 054020
Bruno, G. A. & Christian, J. E. (1960) *J. Amer. Pharm. Ass.* **49**, 560–561
Dalgleish, 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
Dean, R. B. & Dixon, N. B. (1951) *Anal. Chem.* **23**, 636–638
Drum, D. E. & Vallee, B. L. (1970) *Biochem. Biophys. Res. Commun.* **41**, 33–39
Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
Foster, M. A., Hill, H. A. O. & Williams, R. J. P. (1970) in *Chemical Reactivity and Biological Role of Functional Groups in Enzymes* (Smellie, R. M. S., ed.), pp. 187–202, Academic Press, London and New York
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. & Singh, R. M. M. (1966) *Biochemistry* **5**, 467–477
Kuwabara, S., Adams, E. P. & Abraham, E. P. (1970) *Biochem. J.* **118**, 475–480
Malmström, B. G. (1956) *Methods Biochem. Anal.* **3**, 327–352
McPhie, P. (1971) *Methods Enzymol.* **22**, 23–32
Myer, Y. P. & Schellman, J. A. (1962) *Biochim. Biophys. Acta* **55**, 361–373
Newton, G. G. F. & Abraham, E. P. (1956) *Biochem. J.* **62**, 651–658
Sabath, L. D. & Abraham, E. P. (1966) *Biochem. J.* **98**, 11c–13c
Sabath, L. D. & Finland, M. (1968) *J. Bacteriol.* **95**, 1513–1519
Sullivan, W. J. O. (1969) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M., eds.), pp. 423–431, Clarendon Press, Oxford
Vallee, B. L. & Wacker, W. E. C. (1970) in *The Proteins* (Neurath, H., ed.), vol. 5, pp. 94–103, Academic Press, London
Vallee, B. L. & Williams, R. J. P. (1968) *Chem. Brit.* **4**, 397–402
Weber, G. & Anderson, S. R. (1965) *Biochemistry* **4**, 1942–1947