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Fluorescence and Circular Dichroism Studies on the *Streptomyces* R61 DD-Carboxypeptidase–Transpeptidase

PENICILLIN BINDING BY THE ENZYME

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The circular dichroism of the DD-carboxypeptidase–transpeptidase from *Streptomyces* R61 shows in the near u.v. a set of weak extrema at 289nm (positive) and at 282, 275 and 268nm (all negative). In the far u.v. it shows negative extrema at 217–218 and 208nm, crossover at 202nm and a positive maximum at about 194nm. The u.v. absorption of the enzyme shows it to contain tyrosine and tryptophan in approx. 3.4:1 ratio. The enzyme is fluorescent with a maximum emission at 318–320nm. The near-u.v. circular dichroism of the protein is extensively affected by binding of penicillin G, but the far u.v. is unaffected. Binding of the antibiotic also causes quenching of the fluorescence of the enzyme. The latter effect has been used to study the binding of penicillin G to the enzyme and the influence exerted upon it by salts, denaturants and peptide substrates and inhibitors. High-affinity binding of penicillin appears to be comparatively slow and reversible, and can occur under conditions in which the protein is enzymically inactive. The thermal denaturation of the enzyme in guanidinium chloride at pH7 is affected by binding of the antibiotic. The presence of even large concentrations of β-mercaptoethanol neither impaired the activity of the enzyme nor prevented its inhibition by penicillin G or cephalosporin C. A new hypothesis for the molecular mechanism of the interaction of the enzyme with penicillin is proposed.

During the final steps in the biosynthesis of the bacterial cell-wall peptidoglycan (mucopeptide) the terminal D-alanine present in the precursors is removed by two enzymic activities, i.e. carboxypeptidase activity

$$\text{R-D-Ala-D-Ala} + H_2O \xrightarrow{\text{DD-carboxypeptidase}} \text{R-D-Ala} + \text{D-alanine}$$

and transpeptidase activity

$$\underset{\text{Donor}}{\text{R-D-Ala-D-Ala-CO}_2\text{H}} + \underset{\text{Acceptor}}{\text{NH}_2\text{-R}'} \xrightarrow{\text{transpeptidase}} \text{R-D-Ala-CO-NH-R}' + \text{D-alanine}$$

These enzymic activities are membrane-bound in most organisms. However, many *Streptomyces* strains excrete DD-carboxypeptidase in the culture medium and some have been purified to homogeneity or near homogeneity (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1971, 1972). The study of the specificity profile of these DD-carboxypeptidases and the correlation observed between their penicillin sensitivity and that of the strains of *Streptomyces* from which the enzymes were isolated led us to postulate that both enzymic activities, carboxypeptidase and transpeptidase activities, were properties of a single enzyme (Leyh-Bouille *et al.*, 1970 *a*, *b*; 1971). This hypothesis received strong support when it was shown that some soluble, purified DD-carboxypeptidases were able to perform transpeptidation in solution when supplied with the proper carboxyl donor and acceptor substrates (Pollock *et al.*, 1972). The enzyme with which this paper is concerned is the DD-carboxypeptidase–transpeptidase from *Streptomyces* strain R61. It has a molecular weight of 38000 (Frère

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et al., 1973) and it is able to perform transpeptidation to amino acid or peptide acceptors, including those to be expected for the enzyme performing transpeptidation *in vivo* (Perkins *et al.*, 1973). The enzyme is inhibited by penicillin and the antibiotic abolishes both activities simultaneously at the same concentration (Pollock *et al.*, 1972). A widely accepted mechanism for the inhibition of carboxypeptidases and transpeptidases by penicillin and cephalosporins is the so-called "structural analogue' hypothesis (Tipper & Strominger, 1965). This hypothesis assumes that the aminopenicillanic acid part of the penicillin molecule is a structural analogue of the D-Ala-D-Ala terminus of the DD-carboxypeptidase donor substrate. Hence penicillin would be able to bind at the same active site as the donor substrate and could produce a penicilloyl-enzyme by opening of the β-lactam ring and acylation of the enzyme. The penicilloyl-enzyme could not be hydrolysed by water or acceptor substrate and therefore the enzyme would be inactivated. The hypothesis rested mainly on the study of the kinetics of inhibition of DD-carboxypeptidases by the β-lactam antibiotics. However, recently DD-carboxypeptidases have been found that exhibit a non-competitive type of kinetics of inhibition (Leyh-Bouille *et al.*, 1972) and others that are not inhibited by these antibiotics (Leyh-Bouille *et al.*, 1970*a*,*b*). A direct study of the interaction of the transpeptidases with penicillin has not been possible in the past, both from the lack of sufficiently pure, soluble enzyme and of a method sensitive enough to observe the interaction in quantitative terms. The above conditions are now met for the enzyme from strain R61. The purpose of the present paper is to show that circular dichroism in the near u.v. or, better, quenching of the fluorescence of the enzyme, can be used to study the binding of the antibiotic to the enzyme. The results obtained conflict with the 'structural analogue' hypothesis and a new mechanism of inhibition is proposed.

Experimental

Materials

Enzyme. The DD-carboxypeptidase from strain R61 was purified as described by Frère *et al.* (1973). The sample used in most of the work described was 85–90% pure. The concentration of a stock solution of the enzyme was estimated by measuring the number of fringes in the analytical centrifuge. By using this concentration and correcting for a small amount of scattering, a value of $E^{1\%}_{1cm}$ at 280nm of 10 ± 0.5 was estimated. This extinction coefficient was used in other concentration determinations. Crucial experiments were repeated with a sample purer than 95% (Frère *et al.*, 1973), with an identical result. The enzyme solution was stored frozen at −20°C. Because the enzyme showed signs of aggregation immediately after thawing, the thawed enzyme solution was always kept for 2–3h at 0°C before use. The aggregation, however, did not affect the stoicheiometry of antibiotic binding.

DD-Carboxypeptidase activity was measured as described by Nieto *et al.* (1973) with diacetyl-L-lysyl-D-alanyl-D-[^{14}C]-alanine as substrate or by the method of Ghuysen *et al.* (1970). The concentration of substrate was 0.7mM.

Antibiotic and amino acids. Penicillin G was obtained from Glaxo Laboratories Ltd. (Greenford, Middx., U.K.). Tryptophan and lysozyme were from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and trypsin from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.).

The synthesis of diacetyl-L-lysyl-D-alanyl-D-alanine was described by Nieto & Perkins (1971), and of diacetyl-L-lysyl-D-alanyl-D-[^{14}C]alanine and acetyl-D-alanyl-D-glutamic acid by Nieto *et al.* (1973). Glycyl-L-alanine was purchased from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other chemicals were of the best quality commercially available.

Methods

Spectra. U.v. spectra were obtained by using a Unicam SP. 700 double-beam spectrophotometer with automatic slit-width adjustment. Measurements at a single wavelength were performed in a Unicam SP. 500 spectrophotometer.

Circular dichroism was measured with a Jouan Dichrograph mark II (Roussel-Jouan Co., Paris, France). Every spectrum was scanned twice in the near u.v. or three or four times in the far u.v.

Fluorescence emissions were measured at 90° to the exciting beam in an Aminco–Bowman recording spectrofluorimeter with jacketed cell-holder at constant temperature ±0.1°C. The temperature was measured inside the cell with a calibrated thermocouple (W. G. Pye and Co. Ltd., Cambridge, U.K.).

Spectra were recorded manually. Buffered enzyme solution (2ml) having $E^{280}_{1cm} = 0.04-0.05$ was introduced into the cell and to this inhibitors, substrates or other chemicals were added by means of Carlsberg micropipettes. When dilution was higher than 0.2% the value of the fluorescence was corrected accordingly.

pH. The pH value was measured with a model 23A pH-meter (Electronics Instruments Ltd., Richmond, Surrey, U.K.) fitted with a combination electrode and calibrated with standard buffers immediately before use. In the study of the pH-dependence of fluorescence the pH value was adjusted by addition of KOH or HCl solutions of the appropriate concentration (0.5–6M) to avoid dilutions greater than

1–2%. Buffers for the measurement of enzymic activity were all at a final concentration of 0.01M: pH 3–6, sodium acetate; pH 7–9, Tris–HCl; pH 10–11, sodium phosphate.

Results

Tyrosine and tryptophan contents

U.v.-absorption spectrum. The u.v. spectrum of the enzyme in the near u.v. indicated the presence of both tyrosine and tryptophan (Fig. 1). Their relative contents were estimated from the u.v. spectrum in alkali. The method of Bencze & Schmid (1957) gave a value of $s = 16$ corresponding to a ratio tyrosine/tryptophan of 3.4. Some of the tyrosine hydroxyl groups did not titrate below a pH value of about 12. Beyond that pH value titration was time-dependent, being complete in 6 min at pH 13. The final u.v. spectrum in alkali showed a maximum at 290nm, a pronounced shoulder at about 283nm and a minimum at 273nm. The contribution of the time-dependent titration to the total ionized tyrosine absorption at 305nm was about 13%.

Fluorescence. The excitation and emission spectra of the R61 enzyme are shown in Fig. 1. Excitation between 275 and 285nm produced an emission maximum at 318–320nm. The excitation spectra corresponded fairly well with the absorption spectrum (Fig. 1). The fluorescence characteristics were those of tryptophan residues; the short wavelength of the emission maximum suggested a predominantly apolar environment for those residues equivalent to that of tryptophan in butan-1-ol (Konev, 1967). Under identical conditions of measurement, in 0.01M-potassium phosphate buffer, pH 7, and with excitation at 275 or 285nm, the maximum of emission of free tryptophan occurred at 347nm, that of lysozyme at 337nm and that of trypsin at 333nm.

Since prolonged exposure of the enzyme to light at 285nm caused a decrease in the fluorescence intensity (27% in 2.5h), light-exposure was kept to a minimum. However, exposure to light did not affect the activity of the enzyme, its c.d.* spectrum or its affinity for penicillin.

pH-dependence of the fluorescence. The fluorescence of the R61 enzyme was fairly constant in the range pH 4–8, decreasing quite sharply outside this region (Fig. 2). In the basic region the maximum of the emission spectrum remained at 320nm up to about pH 12. The protein could be maintained at pH11.8 for 40min at 25°C without the fluorescence maximum changing in position or the intensity of fluorescence showing any time-dependence. However, at pH12.15 the intensity and position of the maximum in the emission spectrum became time-dependent. After 30min the maximum occurred at 335–337nm and thereafter remained constant. A similar behaviour was observed below pH2. Extensive aggregation of the protein occurred at these extremes of pH as indicated by a great increase in light-scattering. The pH-dependence of the enzymic activity did not agree with that of the fluorescence, especially in the acid region (Fig. 2). At a pH value lower than 5 the

* Abbreviations: c.d., circular dichroism; Ac, acetyl.

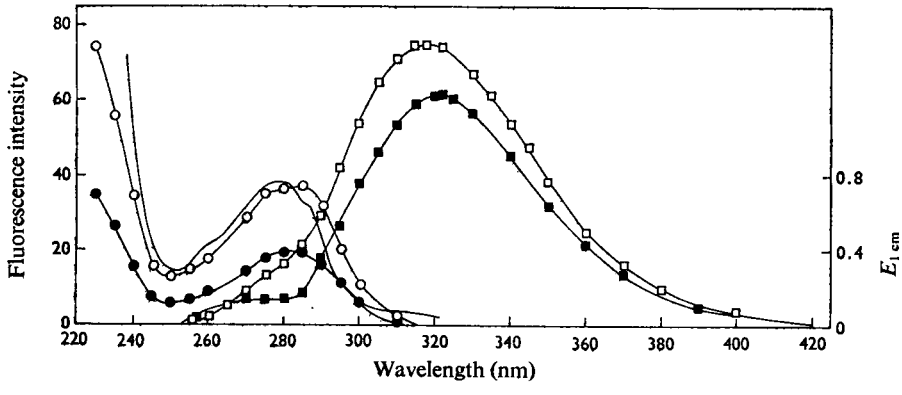

Fig. 1. *Fluorescence spectra of the R61 enzyme*

Excitation spectra corrected for lamp energy output: ○, emission at 320nm; ●, emission at 350nm. Uncorrected emission spectra of the same enzyme when excited at 285nm (□) and 275nm (■). The absorption spectrum of the enzyme (0.71mg/ml) is also shown (——) (right-hand scale). Fluorescence intensity is in arbitrary units. All spectra were obtained at 25°C in 1mM-Tris–HCl buffer, pH7.4. The fluorimeter was set at sensitivity 30, meter multiplier 0.03. For measurement of emission the entrance slits were wide ($\frac{1}{4}$in) and the exit slits narrow ($\frac{1}{32}$in); the reverse arrangement was used to record excitation spectra.

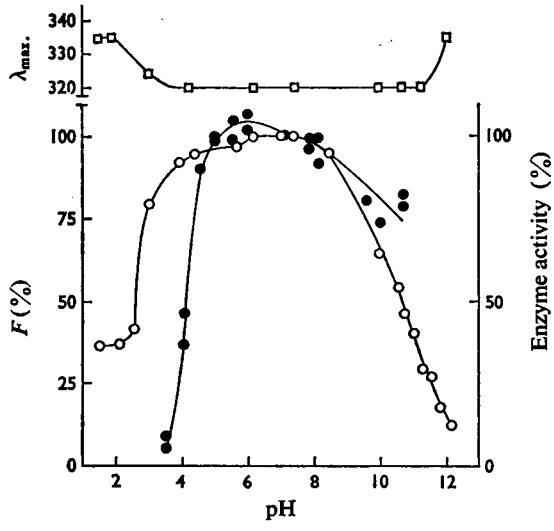

Fig. 2. *pH-dependence of fluorescence and carboxy peptidase activity*

The emission at 320nm was measured at an exciting wavelength of 285nm (○). Carboxypeptidase activity (●) was measured as described in the Experimental section. The fluorescence intensity (F) and enzymic activity at pH7 have been taken as 100. At each pH value complete emission spectra were also obtained as in Fig. 1. $\lambda_{max.}$ is the wavelength at which the maximum of emission occurred.

enzymic activity decayed sharply, whereas the fluorescence was only extensively quenched below pH4. In both the acidic and basic regions the enzymic activity decayed before any red-shift occurred in the emission maximum. The enzyme could be maintained for 24h at pH10 at room temperature without detectable loss of activity upon returning to pH7.

Circular dichroism. The c.d. spectrum of the carboxypeptidase–transpeptidase in the near u.v. presented a set of very weak bands with a positive extremum at 289nm (Fig. 3*a*) and negative extrema at about 282, 275, 268 and 264nm (Fig. 3*a*). In the far u.v. it showed negative extrema at 217–218nm and 208–209nm and a positive extremum at about 194nm (Fig. 3*b*). The negative extremum at 217.5nm and the positive at 194nm suggest contributions from β-structure. Comparison of the c.d. spectra with the computed curves of Greenfield & Fasman (1969) suggested that the enzyme contained approx. 32% of α-helix and 28% of β-structure. These values depend, of course, upon the standard that is used for comparison, but they were consistent with a somewhat compact molecule having rather more β-structure than is often found in globular proteins.

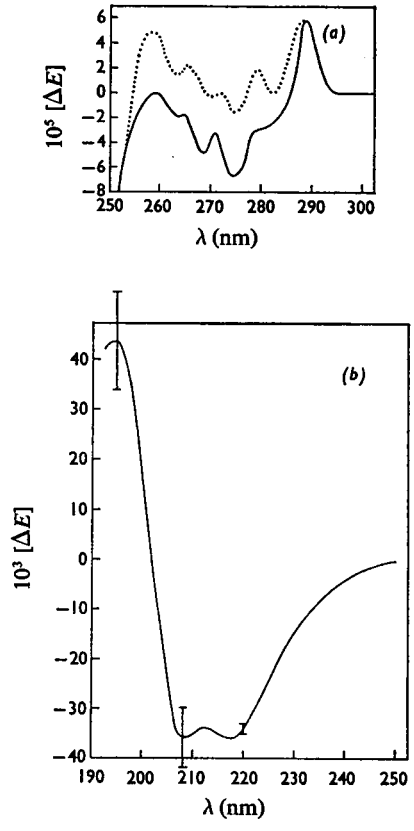

Fig. 3. *Circular dichroism of the enzyme with and without added penicillin*

(*a*) Near u.v. The protein (0.5ml of 0.625mg/ml, corrected for non-enzymic protein) was introduced in a semi-micro 1cm light-path cell and 10μl of 2mM-sodium penicillin G was added by Carlsberg micropipette. The spectrum has been corrected for the dichroism of penicillin below 260nm. Each spectrum was scanned twice. The experiment was carried out in 0.01M-Tris–HCl buffer, pH7.4, at 21±0.1°C. ——, Enzyme alone; · · ·, enzyme saturated with penicillin G. $[\Delta E] = \Delta E/c$, where ΔE is the measured value of the dichroism as difference in absorption for left- and right-hand circularly polarized light in a light-path of 1cm, and c is the concentration of protein in mg/ml. (*b*) Far u.v. The spectrum shown is the average of ten different experiments in which each spectrum was scanned three times. The experiments were carried out in 10mM-, 1mM- or 0.5mM-Tris–HCl, pH7.3–7.4, or in 0.01M-sodium phosphate, pH7.0, at temperatures from 21° to 25°C with light-paths from 1 to 10mm. The concentration of protein ranged from 0.04 to 0.13 mg/ml. A run with a sample of 95% pure enzyme gave the same result. Scan speed was 0.0625nm/s and time-constants were either 4 or 2. The bars across the spectrum represent the maxi-

Interaction between R61 enzyme and penicillin G

Circular dichroism and fluorescence spectra. When sodium penicillin G was added to a neutral solution of the carboxypeptidase–transpeptidase the c.d. spectrum of the enzyme in the near u.v. was extensively altered, except for the positive extremum at 289nm (Fig. 3*a*). The peptide region of the c.d. spectrum in the far u.v. (after correction for the comparatively small dichroism due to the antibiotic) remained unchanged. The interaction between the enzyme and penicillin showed high affinity and no further alterations in the c.d. of the enzyme occurred after 1 mol of penicillin had been added/35000–38000g of enzyme. The overall change in c.d. was, however, too small and the concentrations of enzyme too high to be convenient for quantitative work.

The addition of penicillin G to a solution of the R61 enzyme quenched the fluorescence. The overall quenching appeared to be dependent upon ionic strength. The fluorescence emission at 320nm in the presence of saturating concentrations of penicillin was decreased by 25–30% in 10mM-Tris–HCl buffer, pH7.4, or 10mM-sodium phosphate buffer, pH7, and by 16–18% in 0.19M-sodium phosphate buffer, pH7, or 1M-ammonium acetate. In these experiments the enzyme was not aggregated, as judged by the initial absence of extensive scattering of light. The extent of quenching of the fluorescence at 320nm in 10mM-phosphate buffer, pH 7, was always used to measure binding of the antibiotic (Fig. 4). The curve suggested that maximum quenching occurred when 1 mol of antibiotic had been added to 35000–39000g of R61 enzyme. This value corresponds to the value of 38000 found for the molecular weight in the analytical ultracentrifuge or by gel electrophoresis (Frère *et al.*, 1973). On the assumption that the quenching in fluorescence was directly proportional to the amount of enzyme–penicillin G complex formed, the results yielded an association constant of $1.1 \pm 0.5 \times 10^8$ litre·mol^{-1}. This value was in good agreement with the K_i value of 7.5×10^{-8}M estimated kinetically by Leyh-Bouille *et al.* (1971), taking into account that they carried out their experiments at 37°C, where the affinity for the antibiotic may be smaller.

The binding of cephaloridine and cephalosporin could not be studied by this method because the enzyme required for saturation a concentration of antibiotic of about 0.1mM, which was too highly absorbent for measurements to be made. There was nevertheless an indication that similar binding occurred.

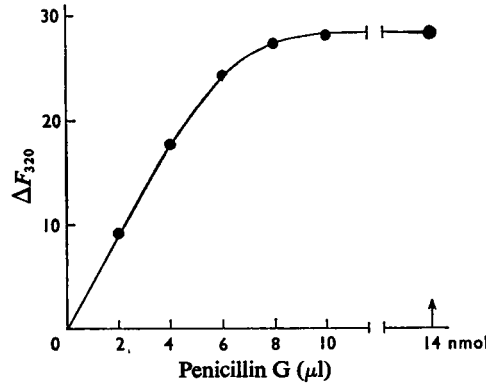

Fig. 4. *Effect of penicillin on the fluorescence of the enzyme*

R61 enzyme (95% pure, 2ml of 26.5μg/ml) was dissolved in 10mM-sodium phosphate buffer, pH7.0, at 25°C. Excitation was at 273nm and the fluorimeter settings were: sensitivity 20, meter multiplier 0.01. Fluorescence intensity (F) was expressed in arbitrary units, the maximum emission being taken as 100. The binding of penicillin G to the enzyme was followed by fluorescence quenching at 320nm (ΔF_{320}). The sodium penicillin G solution (0.232mM) was in 10mM-sodium phosphate buffer, pH7.0. Readings were taken 10min after each addition to allow time for equilibration. After 10μl of penicillin solution had been added, 2.32mM-penicillin (5μl) was added to ensure saturation.

Kinetics of binding of penicillin G. The combination between penicillin and the enzyme reached equilibrium only after about 10min (Fig. 5). In the first 3–4min, when the forward reaction towards enzyme–penicillin complex was dominant, good straight lines in second-order kinetic plots were obtained. The forward reaction rate constant so obtained was $k = 1.5 \times 10^4$ litre·mol^{-1}·s^{-1} in 0.01M-buffer, pH7–7.3 (see also Table 2). At later times the curves in the second-order plots curved in a manner consistent with a reversible reaction.

Reversibility of the interaction between enzyme and antibiotic

Effect of 2-mercaptoethanol. According to the hypothesis of Tipper & Strominger (1965), penicillins and cephalosporins would combine with transpeptidases at the active site for the carboxyl donor substrate and there acylate the protein. Lawrence & Strominger (1970) reported that thiol

mum deviation from the reported curve. The presence of penicillin G did not alter the spectrum after correction for the dichroism of the antibiotic. $[\Delta E] = \Delta E/c$, as in (*a*).

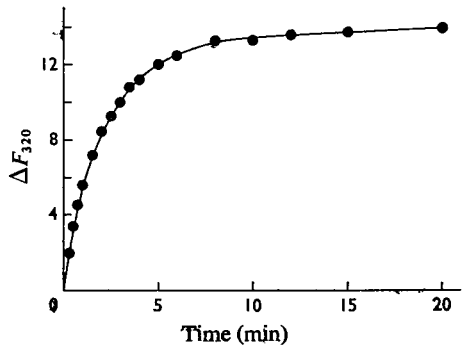

Fig. 5. *Time-course of the binding of sodium penicillin G to the enzyme*

To a solution of the enzyme (7.5×10^{-7} M) in 10mM-sodium phosphate, pH7.0, antibiotic was added to a concentration of 7.6×10^{-7} M and the quenching of the fluorescence at 320nm (ΔF_{320}) was followed with time. The experiment was carried out at 25°C. Other conditions were as described in Fig. 4.

groups on the enzyme from *Bacillus subtilis* were acylated by the antibiotics, and inhibited enzyme could be reactivated by thiol compounds or by hydroxylamine. On the other hand, in the present experiments the R61 carboxypeptidase–transpeptidase recovered full activity when the enzyme, treated with a large excess of penicillin G (100 times the amount required for saturation), was dialysed overnight against 0.01M-buffer, pH7, at 4–22°C. The carboxypeptidase activity of the enzyme was not affected by incubation at 37°C overnight in up to 100mM-2-mercaptoethanol in 0.01M buffer, pH7. When the enzyme was totally inactivated by incubation with 0.1mM-cephalosporin C or 1μM-penicillin G, addition of the thiol compound in the range 1–100mM did not reactivate the enzyme, even after incubation at 25° or 37°C overnight. Conversely, 2-mercaptoethanol (5–100mM) did not protect the enzyme from inactivation by β-lactam antibiotics. It is noteworthy that free penicillin reacted with 2-mercaptoethanol, presumably by opening the β-lactam ring, since after incubation for 1–2h in the absence of enzyme and substrate a product appeared giving a Dnp derivative that was absent from incubations of either 2-mercaptoethanol or penicillin G alone. The Dnp derivative (yellow–reddish) ran very slightly slower than Dnp-Ala on silica gel G t.l.c. with chloroform–methanol–acetic acid (95:5:1, by vol.) as solvent, $E_{max.}$ 375nm in aq. 0.01M-NH_3–methanol (1:1, v/v). Leyh-Bouille *et al.* (1972) also found the reaction between penicillin and the DD-carboxypeptidase from strain K11 to be non-covalent.

Table 1. *Effect of several salts on the fluorescence of the R61 enzyme*

Increasing amounts of a concentrated solution (1–4M) of the salt were added to 2ml of the enzyme in 1mM-Tris–HCl buffer, pH7.3. The fluorescence was corrected for the dilution introduced. The salt solution was pH7 in all cases and the temperature 25°C. K_A is the inverse of the concentration at which half of the maximum quenching of the fluorescence (ΔF) is produced. The fluorescence before addition of salt was taken as 100%.

Salt	K_A (litre·mol^{-1})	ΔF (%)
Ac_2-L-Lys-D-Ala$^-NH_4^+$	5	12
Ac_2-L-Lys-D-Ala-D-Ala$^-NH_4^+$	6–8	11–13
$Ac^-NH_4^+$	4	11
Sodium phosphate	8	25
Guanidinium chloride		22

Effect of salts, and donor and acceptor peptides on the fluorescence of the enzyme and its binding to penicillin G. Several salts were found to quench the fluorescence of the R61 enzyme, but the quenching was so rapid that no time-dependence could be observed as for penicillin. When increasing concentrations of the salt were added to a solution of the enzyme and the quenching was measured, a double-reciprocal plot gave an apparent association constant between the salt and the enzyme (Table 1). Probably the anion was responsible for the interaction, because the quenching was independent of the cation used. The donor substrate included in the table (Ac_2-L-Lys-D-Ala-D-Ala) was no better ligand than phosphate, or than Ac_2-L-Lys-D-Ala, which is neither substrate nor inhibitor of the enzyme. Substances such as glycine or Gly-L-Ala that can function as acceptors in the transpeptidase reaction did not affect the fluorescence of the enzyme. Under the conditions used (25°C, pH7) the gross conformation of the enzyme did not change, as judged by the lack of change in its c.d. spectrum in the far u.v., which was unaffected even by 4M-guanidinium chloride. With increasing ionic strength some aggregation of the enzyme probably occurred, however, as indicated by an increase in light-scattering.

If the enzyme was first saturated with penicillin G and then exposed to salts, the quenching produced by the latter and their apparent K_A was very much decreased. Thus for ammonium acetate K_A became 1.3 litre·mol^{-1} and the further quenching was only 6–7%. Neither Ac_2-L-Lys-D-Ala-D-Ala nor Ac_2-L-Lys-D-Ala, 60mM at pH7, affected the emission spectrum of penicillin G-saturated enzyme, although high concentrations of salts decreased the affinity

of the enzyme for penicillin G (Table 2). The forward rate constants (k_f) for the association of the enzyme and penicillin are also shown in Table 2. It is noticeable that most of the observed decreases in K_A were attributable to a slowing down of the forward reaction. The value of k_r changed relatively little.

The standard donor substrate, Ac_2-L-Lys-D-Ala-D-Ala and the peptide Ac-D-Ala-D-Glu, a competitive inhibitor of the enzyme acting as DD-carboxypeptidase (Nieto *et al.*, 1973), at concentrations fivefold their K_m and K_i respectively, had a small effect, decreasing both k_f and K_A, but the same effect was shown by Ac_2-L-Lys-D-Ala, which was neither substrate nor inhibitor and hence, presumably, did not bind to the donor site of the enzyme. Larger decreases of k_f and K_A occurred in 0.19M-sodium phosphate, 1M-ammonium acetate and 2.66M- and 3.6M-guanidinium chloride. Two types of acceptor molecules in the transpeptidation catalysed by the enzyme, glycine and the dipeptide Gly-L-Ala, had no noticeable effect on K_A or on the rate constants at concentrations that should saturate the acceptor site of the enzyme.

The DD-carboxypeptidase activity of the enzyme was also quite sensitive to ionic strength. Maximum activity occurred at pH7.5, at a buffer concentration of 0.01M, and the activity fell to 30% of maximum at 0.1M (Leyh-Bouille *et al.*, 1971) and was zero in 0.6–0.7M-guanidinium chloride (see below).

Activity of the enzyme in urea and guanidinium chloride solution

The results so far showed that active enzyme, which was therefore capable of binding donor peptide, could bind penicillin and be inhibited by it. In fact binding of penicillin could also occur with high affinity under conditions in which the protein had been made enzymically inactive by two non-specific agents, urea and guanidinium chloride. The experiments were carried out as follows: the enzyme (0.5μg) was incubated without substrate in a final volume of 30μl of 0.01M-sodium phosphate, pH7 (made up in urea or guanidinium chloride at the concentration desired), for 2h at 37°C. Then Ac_2-L-Lys-D-Ala-D-Ala (50nmol) was added and incubation continued for 1h more. The relationship between the amount of D-alanine liberated and denaturant concentration is shown in Fig. 6. Of the two agents, guanidinium chloride was the more efficient in suppressing enzymic activity. Furthermore, the inhibition caused by incubation with urea was reversible, whereas that caused by guanidinium chloride was not. Samples treated with 4–6M-urea at 37°C, in the presence or the absence of 20mM-2-mercaptoethanol, for 2h or overnight were diluted to 0.2M-urea with 0.01M-sodium phosphate buffer, pH7, and then allowed to stand at 4°C or room temperature overnight. When their activity was compared with that of a control identical with the experimental sample but kept in 0.2M-urea during a similar period, a

Table 2. *Association and rate constants for the interaction between the R61 enzyme and penicillin G*

In all cases the volume of the enzyme solution in the agent indicated in the table was 2ml. To this solution a maximum of 10μl of penicillin solution was added and no correction for dilution was required. All experiments were carried out at 25°C. The values of K_A were determined as indicated in the text. The forward rate constant k_f for the reaction penicillin+enzyme $\underset{k_r}{\overset{k_f}{\rightleftharpoons}}$ penicillin–enzyme complex was determined from second-order kinetic plots during the first 3–4min after addition of the antibiotic. k_r was calculated from k_f/K_A.

Solvent	pH	K_A (litre·mol^{-1})	k_f (litre·mol^{-1}·s^{-1})	k_r (s^{-1})	Observations
1–10mM-Tris–HCl	7.3	1.1×10^8	1.1×10^4	1×10^{-4}	Standard
7.5–10mM-Sodium phosphate	7.0	2.2×10^8	1.8×10^4	0.8×10^{-4}	conditions
10mM-Sodium phosphate	7.0	3.0×10^8	1.8×10^4	0.6×10^{-4}	95% pure enzyme
2.66M-Guanidinium chloride	6.8		6.8×10^2		Enzymically
3.6M-Guanidinium chloride	6.8	2.7×10^5	1.3×10^2	0.5×10^{-4}	inactive
1M-$NH_4^+Ac^-$	7.0	2.6×10^5			
0.19M-Sodium phosphate	7.0	2.3×10^7	3.9×10^3	1.7×10^{-4}	
0.57M-Glycine	6.95	2.8×10^8	1.2×10^4	0.4×10^{-4}	
26mM-Gly-L-Ala	6.7	3.6×10^7	1.1×10^4	3.0×10^{-4}	Acceptor substrates
0.112M-Gly-L-Ala	6.75	8.6×10^7	1.1×10^4	1.3×10^{-4}	
57.3mM-Ac_2-L-Lys-D-Ala-D-Ala	6.9	8.5×10^7	5.7×10^3	0.7×10^{-4}	Donor substrate
55.6mM-Ac_2-L-Lys-D-Ala	6.9	1.0×10^8	4.4×10^3	0.4×10^{-4}	Non-substrate, non-inhibitor
19.5mM-Ac-D-Ala-D-Glu	6.9	9.4×10^7	5.1×10^3	0.5×10^{-4}	Competitive inhibitor

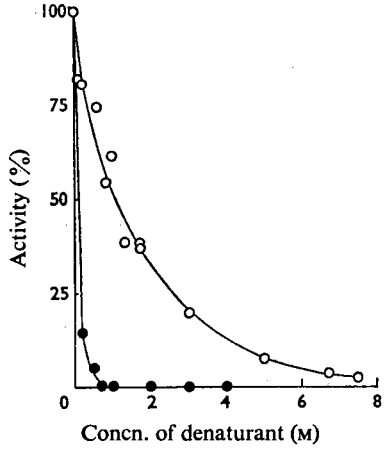

Fig. 6. *Carboxypeptidase activity of the enzyme in the presence of urea* (○) *or guanidinium chloride* (●)

The enzyme was incubated for 2h at 37°C in the desired concentration of denaturant, and then substrate was added and enzymic activity measured as indicated in the Experimental section. The D-alanine liberated was measured by using radioactive substrate or by dinitrophenylation. The presence of even large concentrations of urea or guanidinium chloride did not interfere with the latter estimation. All incubation minxtures were 10mM in sodium phosphate, pH7. The pH value of the solution of denaturant was also adjusted to 7.

recovery of activity of 90–100% was found. The activity of samples similarly treated with 1M-guanidinium chloride did not recover when they were diluted to 0.1M and kept in the cold or at room temperature for 1–2 days. However, as mentioned before, this treatment did not prevent the enzyme from binding penicillin. In 1M-guanidinium chloride the position of the maximum of fluorescence emission remained unchanged and so did the c.d. spectrum in the far u.v., so that evidently no extensive unfolding of the protein occurred. Unfolding started at 37°C only at the highest concentrations of guanidinium chloride used (see below).

Thermal denaturation of the R61 enzyme at neutral pH and the effect of penicillin G

In dilute buffer. When the temperature of a solution of the enzyme in dilute buffer at pH 7.0–7.3 was raised, a linear decrease in the fluorescence intensity was observed up to 50°C (Fig. 7). This linear decay of fluorescence was −1.45%/°C above 20°C. Up to 50°C the quenching was reversible and the enzyme was able to combine with penicillin. Between about 50° and 60°C the decline was more steep and the position

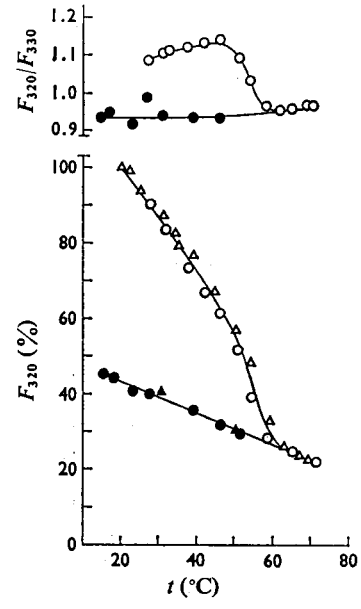

Fig. 7. *Temperature-dependence of the fluorescence of the enzyme*

The fluorescence intensity at 320nm (F_{320}) was measured and the value at 20°C taken as 100%: ○, ●, in 0.01M-sodium phosphate buffer, pH7.0; △, ▲, in 0.01M-sodium phosphate buffer, pH7.0, 35mM in D-alanine. ○, △, Values obtained while the temperature was being raised; ●, ▲, values obtained during re-cooling. For every temperature the fluorescence was followed with time until a stable value was reached.

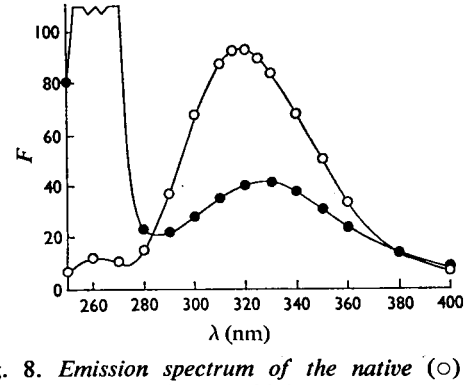

Fig. 8. *Emission spectrum of the native* (○) *and heat-denatured* (●) *enzyme*

The experiment was carried out in 0.01M-sodium phosphate buffer, pH7.0. Both spectra were recorded at 26.5°C. Exciting wavelength was 273nm. The concentration of the enzyme was 28.2μg/ml. *F*, Fluorescence intensity, in arbitrary units.

of the maximum of emission shifted to 329–330nm (Figs. 7 and 8). Beyond 60°C the fluorescence decayed again linearly, but with a lower slope (–0.43%/°C). Above 50°C the changes were not reversible and the cooled protein was unable to combine with penicillin. The effect of temperature was independent of the dilute buffer used and the presence of 35mM-D-alanine, a good acceptor substrate, was also without influence. The heated protein showed strong light-scattering, suggesting extensive aggregation.

The effect of heat-denaturation of the enzyme upon the c.d. spectrum in the far u.v. was surprisingly dependent upon the dilute buffer used (Fig. 9). In very dilute Tris–HCl buffer, pH7.3, the denatured protein had a dichroism apparently dominated by β-structure, whereas in 7.5–10mM-phosphate buffer, pH7, denaturation at 70°C intensified the c.d. extrema and the shape of the spectrum suggested an increase in both α-helix and random structures (Fig. 9).

The thermal denaturation transition was also followed by loss of the enzyme activity. The enzyme was incubated for 1h at the chosen temperature and then brought to 37°C; substrate was added and the activity was measured as usual. Fig. 10 shows the thermal transition, as followed by change in fluorescence at 320nm, c.d. at 208nm and loss of enzymic activity. The agreement between the three methods was very good. Within experimental error, the nature of the buffer did not appear to influence the temperature at which the transition took place.

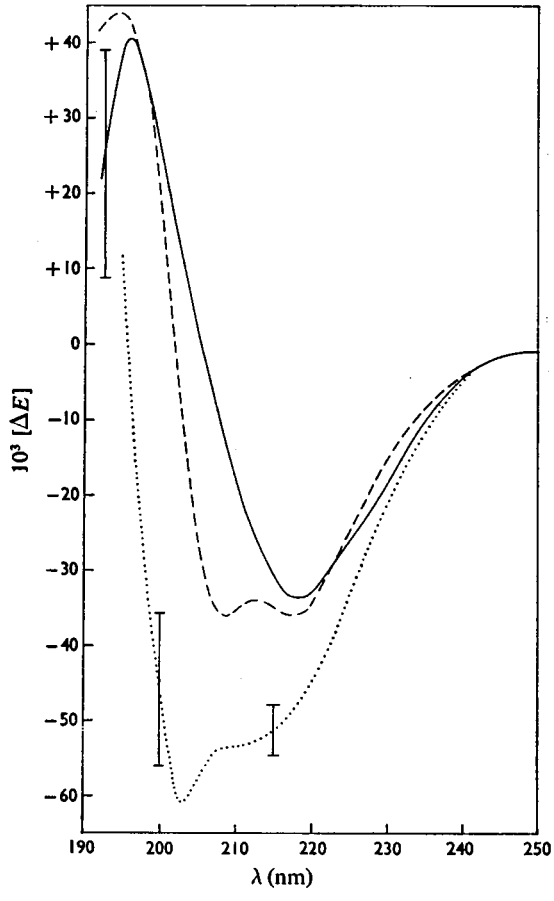

Fig. 9. *Effect of heat-denaturation on the far-u.v. circular dichroism of the enzyme*

––, Native enzyme; ——, heat-denatured enzyme in 0.5–1.0mM-Tris–HCl buffer, pH7.3; ···, heat-denatured enzyme in 7.5–10mM-sodium phosphate buffer, pH7.0. The native protein was introduced to the cell and its c.d. recorded. It was then heated at 70°C and when a stable c.d. value was reached (3–5min) it was cooled to 25°C, at which time the pH values were unchanged. The spectra at 70° and 25°C were identical. Each of the denatured protein spectra is the average of three experiments, and in each of them every spectrum was scanned two or three times. Other conditions were as described in Fig. 3*b*.

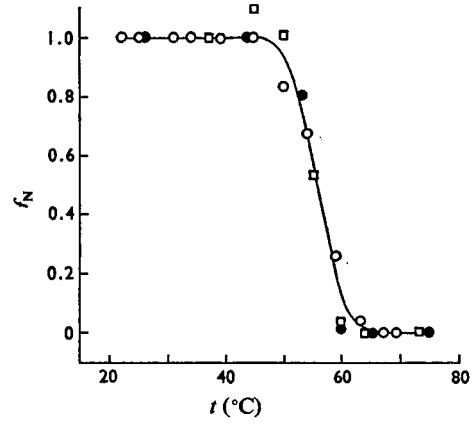

Fig. 10. *Comparison of effects of temperature on circular dichroism, fluorescence and enzymic activity*

Thermal denaturation of the enzyme at neutral pH in dilute buffer was followed by fluorescence at 320nm (○), c.d. at 208nm (●) and loss of enzymic activity (□). The fraction of native protein remaining at a given temperature is $f_N = (P - P_D)/(P_N - P_D)$, where P is the value of the observed parameter at temperature t and P_N and P_D are the values at the same temperature for the native and denatured enzyme respectively. When enzymic activity was the observed parameter, f_N was the ratio of the activity after exposure to the chosen temperature and the activity at 37°C as detailed in the text.

Aggregation and loss of activity were not prevented by 2-mercaptoethanol. In a typical experiment the enzyme was heat-denatured in the presence of 50mM-thiol compound and the strong aggregation and loss of enzymic activity were just as observed in the absence of 2-mercaptoethanol, and were irreversible on cooling. The native enzyme was fully active up to and beyond that concentration of the thiol compound.

Table 3. *Rate constants at several temperatures for the transition from the native to the denatured state of the R61 enzyme alone or saturated with sodium penicillin G*

Fluorescent emission at 320nm was measured. The values of the rate constant were determined from the initial slope of plots of log (% native form) against time. The plot was linear in the first 15 min. An Arrhenius plot of log k versus $1/T$ gave $E = 89$kcal/mol energy of activation for the transition (3.72×10^5J/mol).

Temp. (°C)	$10^{-2}k$ (min^{-1}) Enzyme alone	+Penicillin (1.5mol/mol of enzyme)
51	0.5	
52		0.3
54	1.8	2.2
57.5	7.4	5.8

It was thus very unlikely that intermolecular disulphide bond formation was responsible for the aggregation of the enzyme at neutral pH. Independent experiments had shown the enzymic activity to be insensitive to *p*-chloromercuribenzoate and it seems probable that this enzyme lacks free thiol groups.

When the enzyme was first saturated with an excess of penicillin G and then submitted to heat-denaturation, the antibiotic did not affect, within experimental error, either the transition curve in Fig. 10 for the c.d. spectrum in the far u.v. or the position of the maximum of emission of the denatured species (Fig. 8). However the slope of the linear decay of the fluorescence with temperature (Fig. 7) was consistently affected by penicillin. The antibiotic decreased the slope of the reversible linear part of the curve from −1.45% to −1.20%/°C and increased that of the non-reversible part from −0.43% to −0.57%/°C. The rate of denaturation in the presence and the absence of the antibiotic was measured from the decay of fluorescence at several temperatures in 10mM-sodium phosphate buffer, pH 7.0. First-order plots gave the rates indicated in Table 3, which under these conditions were essentially the same whether penicillin was present or not.

Heat-denaturation in guanidinium chloride. Thermal denaturation can be caused at lower temperatures by the addition of guanidinium chloride. The transition as followed by c.d. and fluorescence is shown in Fig. 11(*a*). The heat-denatured enzyme was still considerably aggregated in 1.7M-guanidinium chlor-

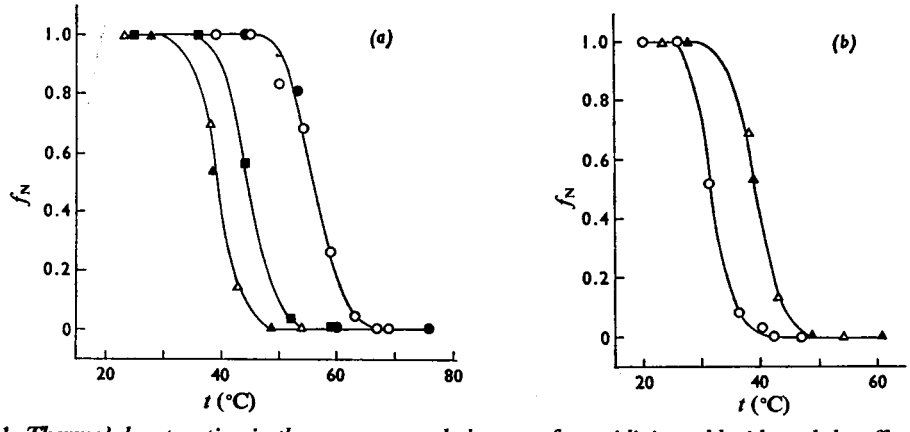

Fig. 11. *Thermal denaturation in the presence amd absence of guanidinium chloride and the effect of penicillin*

Thermal denaturation of the enzyme at pH 7, followed by fluorescence (open symbols) and c.d. (filled symbols). (*a*) The experiments were carried out in the absence of guanidinium chloride (○, ●) or in 1.7M-(■) and in 3.6M-guanidinium chloride (△, ▲). All solutions of enzyme were 10mM in sodium phosphate. Fluorescence was excited at 275nm and emission measured at 320, 330 and 340nm. c.d. was measured at 220nm. (*b*) Influence of penicillin G on the thermal transition of the enzyme in guanidinium chloride. △, ▲, Enzyme in 3.6M-guanidinium chloride; ○, enzyme saturated with antibiotic in 3.6M-guanidinium chloride. The other experimental conditions are as given in Fig. 11(*a*).

Table 4. *Rate constants for the transition from the native form to the denatured form in* 3.6M-*guanidinium chloride of the R*61 *enzyme alone or saturated with penicillin*

The values of k were obtained as described in Table 3. An Arrhenius plot of k against $1/T$ gave $E_{activation}$ 23.4kcal/mol (98.2kJ) for penicillin-saturated and 33.9kcal/mol (142.5kJ) for untreated enzyme.

Temp. (°C)	10^3k (min^{-1}) Enzyme alone	+Penicillin (13mol/mol of enzyme)
31		10.2
36		18.4
37	10.2	
44	40.3	

ide but not in concentrations of 3M or higher. Unfolding and exposure to solvent of the fluorophores was reflected in the quenching of the fluorescence intensity and the position of the maximum of tryptophan emission, now occurring at 345nm.

If the enzyme was first saturated with penicillin G in 3.6M-guanidinium chloride and then heat-denatured (Fig. 11*b*), the transition occurred at a lower temperature ($f_N = 0.5$ at 31°C) than for the untreated protein ($f_N = 0.5$ at 39°C). Also, the penicillin-treated protein denatured faster as shown by the rate constants in Table 4. As expected, the protein after being heat-denatured in guanidinium chloride did not combine with penicillin G.

Discussion

*Properties of the R*61 *enzyme*

There are good reasons to believe that the carboxypeptidase–transpeptidase excreted into the medium by *Streptomyces R*61 is the soluble form of the membrane-bound enzyme. Some of its properties found in this work also support this idea. Thus the protein had a strong tendency to aggregate under many circumstances, but this aggregation was sensitive to ionic strength, pH and, indeed, temperature.

The enzyme was fairly resistant to denaturation, either by pH, heat or denaturing agents at neutral pH. As with many other proteins, some of the tyrosine residues titrated only after unfolding of the protein. This was the most probable explanation for their lack of titration below pH 12 and the time-dependent titration beyond this pH value. The fluorescence decayed sharply below pH3 and beyond pH8.5. The loss of protein fluorescence in the alkaline region is commonly attributed to energy transfer from excited tryptophan residues to ionized tyrosine residues acting as energy sinks (Chen *et al.*, 1969). The decrease in fluorescence in the acidic region seemed to be related to unfolding and/or refolding of the protein and exposure of the tryptophan residues to a more polar environment, as judged by the red-shift of the emission maximum. However, these changes took place only at a pH value lower than that at which enzyme activity had almost disappeared. In the pH-denatured protein the maximum of emission occurred at 335–336nm (Fig. 2), which still represented a blue shift of about 10nm compared with most other unfolded proteins (Konev, 1967). The same was even more true of the heat-denatured protein in dilute buffer, where the emission occurred at 330nm (Fig. 8). This suggested that complete unfolding of the protein was never achieved, and c.d. confirmed this point.

The enzyme was not unfolded by 4M-guanidinium chloride. Unfolding could be induced by heating and then it occurred at much lower temperatures than with the enzyme in dilute buffer (Fig. 11). The maximum of emission in this case shifted to 345nm, a usual value for tryptophan residues in proteins exposed to solvent.

*Interaction between penicillin G and the R*61 *enzyme*

Quenching of fluorescence proved a very convenient method to study the interaction of the enzyme with penicillin. The association constant of the interaction was very high and comparable with the inhibition constant determined kinetically. The quenching of fluorescence induced by penicillin G showed a marked time-dependence (Fig. 5). The time-dependence of the binding of penicillin pointed either to a slow reaction with formation of a covalent bond or to a conformational change in the protein subsequent to the initial binding. Because of the ready reversibility of the reaction, the latter explanation was probably true. The alteration of c.d. in the near u.v. pointed to the same conclusion, since recent theoretical studies by Chen & Woody (1971) and Hooker & Schellman (1970) showed that the c.d. bands in the near u.v. of tyrosine were strongly conformation-dependent. An effect of penicillin on the conformation of the enzyme was also indicated by its influence on the temperature of transition in the heat-denaturation in 3.6M-guanidinium chloride (Fig. 11*b*). However, the conformational change was probably not very extensive because it was not reflected in the far-u.v. dichroism of the protein. Other facts also pointed to the ability of this enzyme to undergo limited conformational change. Thus the enzymic activity was lost in urea or guanidinium chloride without the far-u.v. c.d. being altered. A comparison of the pH–activity and pH–fluorescence profiles showed that at

low pH values activity decayed before any extensive alterations in the enzyme conformation occurred, but of course the loss of enzymic activity could be related to protonation of critical groups in the active centre (i.e. β- or γ-carboxyl groups) rather than to conformational change. Binding of penicillin at the acceptor site seemed unlikely, since good acceptors for transpeptidation had no effect on the interaction between the enzyme and the antibiotic.

High concentrations of anions decreased the affinity of penicillin for the enzyme, and donor substrate and peptide competitive inhibitors that are donor analogues behaved like other anions (Table 2). The effect of the standard donor substrate, Ac_2-L-Lys-D-Ala-D-Ala, was no different from that of Ac_2-L-Lys-D-Ala, which was neither substrate nor inhibitor of the enzyme. Anions also caused quenching of the fluorescence of the enzyme (Table 1), but only at very high concentrations and too rapidly to show time-dependence by the present methods. Salts at a concentration higher than an ionic strength of 0.01 decreased the enzymic activity quite sharply (Leyh-Bouille *et al.*, 1971). The linearity of a double-reciprocal plot of salt concentration and fluorescence quenching suggested that salts affected penicillin binding by competing for the same binding site for anions.

Both penicillin and donor substrate combine with the enzyme, but not necessarily at the same site. The quenching of the fluorescence of the enzyme caused by peptide donor substrate seemed to bear no relation to the formation of enzymically productive complex because (*a*) similar quenching was caused non-specifically by salts and a non-inhibitor, non-substrate peptide, whereas binding of donor substrate is very specific (Leyh-Bouille *et al.*, 1971; Nieto *et al.*, 1973), and (*b*) the K_A for the association of the enzyme with the donor substrate as estimated by quenching of fluorescence (Table 1) was very different from the inverse of the K_M determined kinetically, which was probably the dissociation constant of the enzyme–donor substrate complex (Nieto *et al.*, 1973). Thus although salts interfered with both binding of penicillin and enzymic activity they probably did so at different sites. This idea was supported by the following facts. (*a*) Penicillin was bound at concentrations of guanidinium chloride at which the enzymic activity was abolished and could not be recovered directly by dilution of the denaturant. (*b*) Peptide–donor substrate or competitive inhibitor at a concentration fivefold their K_M or K_I respectively had a very small effect on penicillin binding to the enzyme, and this effect was very similar to that of a peptide that was neither substrate nor inhibitor of the enzyme and of other anions (Table 2). (*c*) Although salts affected both penicillin binding and enzymic activity they did not do so to the same extent and at similar concentrations. Thus maximum enzymic activity occurred in the range of ionic strength from 0 to 0.025, with a sharp optimum around 0.01 (Leyh-Bouille *et al.*, 1971), whereas penicillin binding was only noticeably affected beyond 0.1 M and did not show any optimum of ionic strength between 0 and 0.01.

A number of observations indicate that penicillins are not recognized as analogues of a D-alanyl-D-alanine *C*-terminus by biological systems. Thus peptides with that *C*-terminus were neither substrates nor inhibitors of *Bacillus cereus* penicillinase, nor did they induce production of the enzyme by inducible strains (M. R. Pollock & H. R. Perkins, unpublished observations). Also, precisely the *Streptomyces* DD-carboxypeptidases that show a greater specificity of recognition and affinity for donor substrate are either insensitive to the penicillins (*S. albus* G, Leyh-Bouille *et al.*, 1970*a*,*b*) or are inhibited in a non-competitive manner (strain R39, Leyh-Bouille *et al.*, 1972). Finally a DD-carboxypeptidase from *Bacillus megaterium* QMB 1551 that was barely sensitive to cloxacillin was apparently stimulated rather than inhibited by penicillin G (Wickus & Strominger, 1972).

We now suggest a new mechanism for the inhibition of carboxypeptidases and transpeptidases that accounts for the known experimental facts. Studies of inhibition of *Streptomyces* DD-carboxypeptidases by many synthetic peptides (Nieto *et al.*, 1973) led to the conclusion that the enzyme binds to the donor substrate when the amide bond in its *C*-terminal dipeptide has a *cis* configuration. The side chain of the L-residue preceding this *C*-terminal dipeptide would then induce a change in the conformation of the enzyme, resulting in the distortion of the *cis*-amide bond to a conformation intermediate between *cis* and *trans*. This would place the peptide linkage on the top of an energy barrier of some 30–40kJ, and the change in conformation induced in the enzyme has to supply it (Nieto *et al.*, 1973). On the other hand, the interaction of the enzyme with penicillin produced a limited conformational change in the enzyme with a Gibbs free energy change of some 45kJ at 25°C. This might or might not prevent the enzyme from still recognizing and binding the substrate, but if one assumes that the enzyme would first have to return to its normal conformation before it could effect hydrolysis, an energy barrier of 35+45 kJ would have to be surmounted. This is more than twice as much as in the absence of the antibiotic and presumably the enzyme is unable to overcome it. Thus inhibition by penicillin would occur through 'freezing' of the conformation of the enzyme.

The possible existence in transpeptidases of binding sites for penicillin that are independent of the binding sites for their substrates poses other problems, such as why such sites should exist at all. Allosteric control sites would be one possible answer.

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