Biochem. J. (1978) **169**, 381–388
Printed in Great Britain

Preferential Nitration with Tetranitromethane of a Specific Tyrosine Residue in Penicillinase from *Staphylococcus aureus* PC1

EVIDENCE THAT THE PREFERENTIALLY NITRATED RESIDUE IS NOT PART OF THE ACTIVE SITE BUT THAT LOSS OF ACTIVITY IS DUE TO INTERMOLECULAR CROSS-LINKING

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(*Received* 1 *July* 1977)

1. Nitration of tyrosine residues of staphylococcal penicillinase was accompanied by a partial loss of enzymic activity, which was not readily explained by nitration of a single residue. 2. Loss of activity correlated with low recovery of tyrosine plus nitrotyrosine, which was consistent with cross-linking. 3. The fraction of treated enzyme that was eluted from Sephadex G-75 earlier than native penicillinase was similar to the fraction of enzyme activity lost. Protein eluted in positions corresponding to monomer, dimer and higher oligomers respectively showed major bands in corresponding positions in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, indicating that the increase in molecular weight was due to intermolecular cross-linking. Monomeric enzyme containing up to 4 mol of nitrotyrosine/mol retained full catalytic activity. Dimeric enzyme retained 50% of normal activity, whereas higher oligomers retained an average of 8–15% of normal activity. 4. Monomeric enzyme isolated after treatment with equimolar tetranitromethane was nitrated predominantly at tyrosine-72. 5. Reaction of reduced nitrated monomer with 1,5-difluoro-2,4-dinitrobenzene gave a monomeric, apparently cross-linked product with full catalytic activity. 6. It is concluded that tyrosine-72 plays no part in the active site. Its preferential nitration may be due to its being insufficiently exposed to be available for intermolecular cross-linking. This property may make it useful for attachment of a reporter group.

Penicillinase (penicillin amido-β-lactam hydrolase, EC 3.5.2.6) from *Staphylococcus aureus* PC1 was first isolated and characterized by Richmond (1963) as consisting of a single polypeptide chain. The complete amino acid sequence (calculated mol.wt. 28823) has been described by Ambler (1975), who also showed that treatment of the enzyme with equimolar tetranitromethane at pH 8.7 led to a decrease in enzyme activity to about 50% of normal, but that even a 20-fold excess of the reagent did not decrease the activity to less than 10% of normal. Most of the 3-nitrotyrosine found after digestion with trypsin followed by chymotrypsin was in the peptide from residues 67–72, including tyrosine-72. These results are in line with work on other penicillinases; a preferentially nitrated tyrosine residue has been found in a similar region of sequence and in a corresponding position in the polypeptide chain (Meadway, 1969; Scott, 1973). It is thus tempting to suggest that the residue has an important function in penicillinase activity.

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In addition to ready reaction between tyrosine residues and tetranitromethane, the reagent has been found to react at high concentration with tryptophan, cysteine, methionine and histidine residues (Cuatrecasas *et al.*, 1968; Sokolovsky *et al.*, 1969). However, the first two residues are absent from penicillinase. Also, under the conditions used by Ambler (1975) and in the present work it is likely that only tyrosine residues would react. In addition to nitration, polymerization has been observed in several proteins after treatment with low molar ratios of tetranitromethane, e.g. collagen and γ-globulin (Doyle *et al.*, 1968), ovotransferrin (Williams & Lowe, 1971), L-asparaginase (Shifrin & Solis, 1972), ribosomal proteins (Shih & Craven, 1973) and papain (Tsukamoto & Ohno, 1974). In trypsinogen and trypsin (Vincent *et al.*, 1970), insulin (Boesel & Carpenter, 1970) and human lysozyme (Fawcett *et al.*, 1971) polymerization was associated with low recovery of tyrosine plus nitrotyrosine in amino acid analysis compared with the tyrosine content of untreated protein. Polymerization has been interpreted as tetranitromethane-facilitated reaction between tyrosine residues as an alternative to nitration.

The cross-linking reaction has been suggested to be analogous to the formation of Pummerer's ketone, which is obtained by reaction of *p*-cresol and tetranitromethane (Bruice *et al.*, 1968); an alternative suggestion has been formation of biphenyl cross-links between tyrosine residues (Williams & Lowe, 1971), supported by the observation of Shifron & Solis (1972) that L-asparaginase retained an unchanged number of titratable phenolic hydroxyl groups after cross-linking. In either case, cross-linking would be expected to be accompanied by conversion of tyrosine into a form that would not be recovered either with tyrosine or with nitrotyrosine in amino acid analysis.

In view of the variety of results obtained with other proteins, it was appropriate to attempt to fractionate treated penicillinase. At one extreme, the partially active enzyme could consist of a mixture of active and inactive molecules. Alternatively, at the other extreme it could be a homogeneous population of molecules with equal partial activity. In the present work, tetranitromethane-treated penicillinase has been fractionated by gel-filtration chromatography and by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Partial loss of enzyme activity on treatment was accompanied by polymerization, and substantially nitrated monomeric penicillinase was shown to possess normal catalytic activity. Lightly nitrated, fully active penicillinase monomer was nitrated predominantly at tyrosine-72. The activity of this preparation was unaffected when a bulky cross-linking reagent, 1,5-difluoro-2,4-dinitrobenzene, was reacted with it.

Materials and Methods

Materials

Trypsin (treated with diphenylcarbamoyl chloride) and chymotrypsin were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Tetranitromethane was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Fluorescamine was from Pierce and Warriner, Chester CH1 4EF, U.K. Benzylpenicillin (potassium salt), acrylamide, *NN'*-methylenebisacrylamide, 1,5-difluoro-2,4-dinitrobenzene and other chemicals (AnalaR grade when obtainable) were from BDH Chemicals, Poole, Dorset, U.K.

$(NH_4)_2SO_4$/acetate buffer, pH5.0, contained 0.45M-$(NH_4)_2SO_4$, 0.5mM-EDTA (disodium salt) and 50mM-sodium acetate/27mM-acetic acid.

Penicillinase preparation

Penicillinase was isolated and purified from the supernatant fraction of centrifuged liquid cultures of *Staphylococcus aureus* PCI (Richmond, 1963; Ambler, 1975) as described by Robson & Pain (1976). The product was concentrated by vacuum dialysis to a concentration of approx. 10mg of protein/ml and dialysed against $(NH_4)_2SO_4$/acetate buffer, pH5.0. In this solution it was stable for several months. Preparations had a specific activity of 300–320 units/mg of protein [the unit of enzyme activity is defined as the amount of enzyme requiring the addition of 10mM-NaOH at a rate of 1μmol/min to maintain a constant pH (6.9–7.0) in a pH-stat cell containing 0.1M-NaCl and 1.4mM-benzylpenicillin at 25°C]. Protein concentration was determined by measuring the $A_{276.5}$, assuming that $A^{1\%}_{1cm} = 6.87$ (Robson & Pain, 1976).

Treatment of penicillinase with tetranitromethane

Except where otherwise stated, penicillinase was dialysed at 4°C against $(NH_4)_2SO_4$/acetate buffer, pH5.0, and adjusted to pH8.7 with 1M-NaOH at room temperature (20–25°C) immediately before addition to 1ml of solution of 2–5μl of an ethanolic solution of 5–500mM-tetranitromethane followed by incubation for 30min at room temperature (20–25°C).

Determination of protein concentration of nitrated penicillinase

To determine protein concentration after treatment of penicillinase with tetranitromethane, a correction was made to the $A_{276.5}$ to allow for the effect of nitration of tyrosine, the extent of which was determined separately by amino acid analysis. It was assumed that $\varepsilon_{276.5}$ in native penicillinase was equal to the sum of contributions from tyrosine residues, for which $\varepsilon_{276.5}$ was assumed to be 1.34×10^3 litre·mol^{-1}·cm^{-1} (Beaven & Holiday, 1952), and contributions from other residues. On this basis tyrosine accounted for 87.5% of the observed absorption. It was further assumed that nitration affected only the contribution from tyrosine residues by increasing $\varepsilon_{276.5}$ to 5.49×10^3 litre·mol^{-1}·cm^{-1}. This was estimated from the observed ratio of $A_{276.5}/A_{360}$ for 3-nitrotyrosine (1.97) in $(NH_4)_2SO_4$/acetate buffer, pH5.0, and the published value of ε_{360} for α-*N*-acetyl-3-nitrotyrosine at low pH (Riordan *et al.*, 1967).

Amino acid analysis

Freeze-dried samples, containing up to 50nmol of protein or peptide, were sealed *in vacuo* with 2ml of twice-redistilled 6M-HCl and 2–3mg of phenol, which was added to minimize loss of tyrosine (Sanger & Thompson, 1963). After 24h at 110°C, HCl was removed by rotary evaporation at 45°C. Amino acid analysis was carried out with a mark 4 Floor Model amino acid analyser (Locarte Scientific Instruments, London W12 9RT, U.K.).

The nitrotyrosine content of hydrolysed penicillinase was estimated relative to the sum of recoveries of phenylalanine, histidine and arginine, assuming this sum to represent 13 residues/penicillinase molecule. Recovery of these residues was close to the ratio 7:2:4, as expected from the amino acid sequence of penicillinase. On this basis the apparent recovery of tyrosine from native penicillinase was typically 14 residues/molecule rather than 13 residues as expected from the complete sequence (Ambler, 1975).

Identification of nitrated peptides

Peptides were obtained by digestion with trypsin and chymotrypsin and fractionated by gel filtration on Sephadex G-25 and high-voltage paper electrophoresis at pH6.5 as described by Ambler (1975), except that for high-voltage electrophoresis the coolant was Isopar L (Rutpen, Reading, Berks., U.K.) containing approx. 1% (v/v) pyridine. Peptides were detected on paper by lightly spraying with fluorescamine (Vandekerckhove & Van Montagu, 1974).

Fractionation of tetranitromethane-treated penicillinase by gel filtration

After incubation with tetranitromethane as described above, the reaction mixture was applied to the top of a column (2.2×54cm) of Sephadex G-75, which was equilibrated and eluted at room temperature with $(NH_4)_2SO_4$/acetate buffer, pH5.0, at a flow rate of 21ml/h.

Fractions (1.5ml) were collected. Elution volume was expressed as V/V_0, where V_0 (void volume, 81ml) was measured in a separate experiment by using Blue Dextran. To calibrate the column, samples of lysozyme, untreated penicillinase and bovine serum albumin (mol.wt. 14300, 28823 and 69000 respectively) were passed through the column in separate experiments, yielding values of V/V_0 of 1.98, 1.52 and 1.20 respectively.

Results and Discussion

Inactivation of penicillinase by tetranitromethane

Penicillinase did not retain full catalytic activity after dialysis in buffers similar to those used by Ambler (1975) in his investigation of tyrosine reactivity. Thus, after dialysis of penicillinase (6mg/ml) for 24h at 4°C against 50mM-Tris/29mM-HCl, pH8.0, enzyme activity had fallen by 63%. The presence of Na_2SO_4, which stabilized the enzyme against denaturation by urea (C. Mitchinson & R. H. Pain, unpublished work) led to improved retention of activity: after dialysis against 50mM-Tris/10.3mM-HCl/0.1M-Na_2SO_4, pH8.7, activity had fallen by 34%. When enzyme in $(NH_4)_2SO_4$/acetate buffer, pH5.0, was adjusted to pH8.7 with 1M-NaOH at room temperature, full activity was observed during the next 60min. This last procedure was adopted for most of the experiments involving tetranitromethane treatment. Fig. 1 shows the enzymic activity immediately after treatment with tetranitromethane at various concentrations. Similar results were obtained with the partially active preparations obtained by dialysis under the conditions described above. As observed by Ambler (1975) inactivation was less than 100%, even after treatment with a 10- or 20-fold molar excess of reagent. To check that the inactivation process had finished after 30min, the extent of inactivation was measured as a function of time after treatment with tetranitromethane as described in the Materials and Methods section, with penicillinase concentrations of 0.6mg/ml or 6mg/ml and with a 1- or 10-fold molar excess of tetranitromethane. In each case, there was an initial fall in enzyme activity and no further fall after 10–12min. In control incubations, to which ethanol was added in place of an ethanolic solution of tetranitromethane, there was no loss of activity during the total incubation period of 30min. These observations are in line with those of previous workers (e.g. Cuatrecasas *et al.*, 1968), who found that the reaction between tetranitromethane and tyrosine residues was rapid under comparable conditions. A reaction time of 30min was used routinely in subsequent experiments.

The extent of inactivation increased with increasing extent of nitration of tyrosine residues (Fig. 2*a*). The complex shape of this curve suggests that loss of

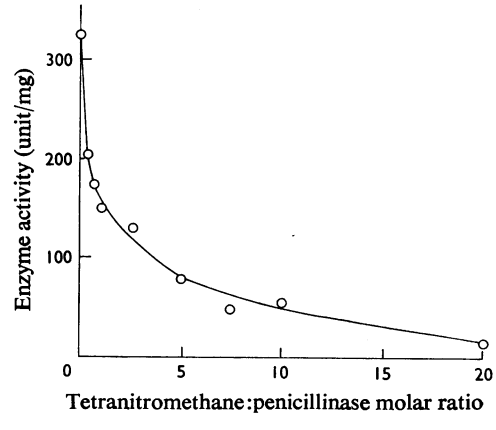

Fig. 1. *Activity of penicillinase as a function of concentration of tetranitromethane*

Penicillinase (7.9mg/ml) was incubated with tetranitromethane and enzymic activity was measured at the end of the incubation period as described in the Materials and Methods section.

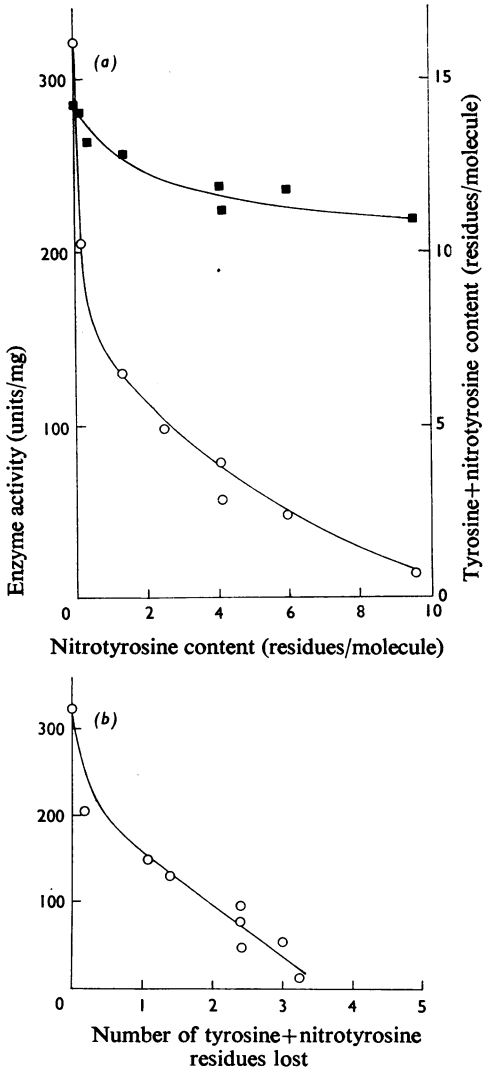

Fig. 2. *Inactivation as a function of extent of nitration* (*a*) Specific activity of penicillinase after treatment with tetranitromethane (the same data as in Fig. 1) (○) and total recovery of tyrosine+nitrotyrosine (■) as a function of nitrotyrosine content determined by amino acid analysis after acid hydrolysis are shown. (*b*) Specific activity after nitration (the same data as Fig. 1) as a function of loss of tyrosine+nitrotyrosine (relative to tyrosine recovered in amino acid analysis of untreated penicillinase) is shown. For further details see the text.

activity was not simply related to nitration of a single tyrosine residue. The data of Fig. 2(*a*) also show that total recovery of nitrotyrosine plus tyrosine after acid hydrolysis fell as the extent of nitration increased. The results shown in Fig. 2(*b*) indicate an approximate correlation between decrease in specific activity and low recovery of tyrosine plus nitrotyrosine. This suggests that loss of activity may be caused by covalent cross-linking through tyrosine, rather than by nitration.

Fractionation of tetranitromethane-treated penicillinase by gel-filtration

Penicillinase treated with tetranitromethane and fractionated on Sephadex G-75 as described in the Materials and Methods section gave the elution patterns shown in Fig. 3. A yellow band, presumably nitroformate, was considerably retarded on Sephadex and is not illustrated. A control sample of enzyme that had been treated in an identical way except for omission of tetranitromethane was eluted as a major peak ($V/V_0 = 1.52$), after a minor peak which contained 7% of the total $A_{276.5}$. The elution profiles of tetranitromethane-treated enzyme each included a peak in a position corresponding to the main peak of the control, and with an elution volume identical with that of monomeric penicillinase. In addition, the elution profile contained a shoulder the centre of which corresponded to the elution volume expected for penicillinase dimer ($V/V_0 = 1.24$) and a peak whose elution volume was close to the exclusion limit ($V/V_0 = 1.02$), indicating a molecular weight greater than that of dimer. Calculation of the proportion of protein in each part of an elution profile required correction for the increase in $\varepsilon_{276.5}$ that occurs on nitration of tyrosine (see the Materials and Methods section). When this was done for pooled fractions from each gel-filtration experiment (Table 1), material eluted earlier than monomeric penicillinase was estimated to account for 30, 68 and 79% in penicillinase treated with a 1-, 2- and 10-fold molar excess of tetranitromethane respectively. After tetranitromethane treatment, but before Sephadex fractionation, these samples had respectively lost 33, 60 and 77% of their initial activity, closely correlating with the total production of aggregate.

Table 1 shows that pooled monomer fractions from each sample of tetranitromethane-treated enzyme had a specific activity closely similar to that of untreated penicillinase. However, dimer fractions had a specific activity of not greater than 50% of normal, and higher-molecular-weight fractions had an activity of 8–15% of normal. Each of the three regions of the elution pattern contained some nitrotyrosine, the content per mol of penicillinase being as great or greater in monomer than in higher-molecular-weight material. After treatment with a 10-fold molar excess of tetranitromethane, the monomer fraction contained almost 4 mol of nitrotyrosine/mol of enzyme, but retained normal catalytic activity. At both concentrations of tetranitromethane, recovery of tyrosine

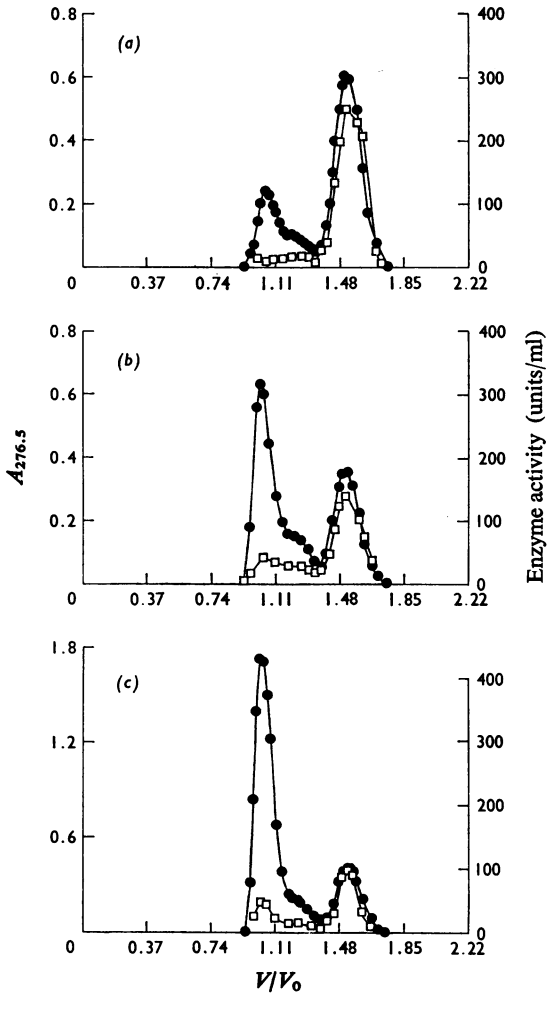

Fig. 3. *Fractionation of tetranitromethane-treated penicillinase on Sephadex G-75*
Samples of penicillinase (11.3mg) were allowed to react with (*a*) 1-, (*b*) 2- or (*c*) 10-fold molar excess of tetranitromethane and then fractionated on a Sephadex G-75 column (for details see the Materials and Methods section). □, Enzymic activity; ●, $A_{276.5}$.

plus nitrotyrosine in the monomer fraction was the same as the tyrosine content of untreated penicillinase. Conversely, a low recovery was observed in higher-molecular-weight fractions, the recovery being lower in multimer than in dimer. This result is consistent with involvement of tyrosine in chemical cross-linking, as well as in nitration.

Table 1. *Activity and extent of nitration of tetranitromethane-treated penicillinase after fractionation on Sephadex G-75*
After elution from Sephadex G-75 (Fig. 3) pooled fractions were assayed for penicillinase activity and $A_{276.5}$ and then exhaustively dialysed against water before amino acid analysis. The extent of nitration found was used to correct $A^{1\%}_{1cm}$ and hence to calculate specific activity (for details, see the Materials and Methods section). Abbreviation: NO_2Tyr, nitrotyrosine.

	Multimer				Dimer				Monomer			
Range of V/V_0 ...	0.96–1.13				1.15–1.35				1.37–1.69			
		Amino acid content (mol/mol of enzyme)				Amino acid content (mol/mol of enzyme)				Amino acid content (mol/mol of enzyme)		
Tetranitromethane added (mol/mol of enzyme)	Specific activity (units/mg)	Tyr	NO_2Tyr	Total Tyr $+NO_2Tyr$	Specific activity (units/mg)	Tyr	NO_2Tyr	Total Tyr $+NO_2Tyr$	Specific activity (units/mg)	Tyr	NO_2Tyr	Total Tyr $+NO_2Tyr$
1	27.7	11.30	0.29	11.59	122.7	11.25	<0.10	11.25	304	13.87	0.28	14.15
2	53.5	8.31	0.36	8.67	139.3	10.0	0.72	10.72	354	11.58	1.56	13.14
10	28.12	5.90	2.03	7.93	80.9	7.65	2.36	10.01	300	10.20	3.80	14.00

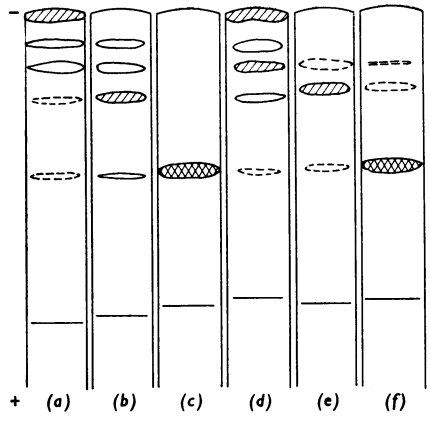

Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of tetranitromethane-treated penicillinase after fractionation by column chromatography with Sephadex G-75*

Samples of multimer (*a* and *d*), dimer (*b* and *e*) and monomer (*c* and *f*) fractions were analysed by gel electrophoresis (10% gels) as described in the text after treatment of penicillinase with either equimolar (*a–c*) or a 10-fold molar excess (*d–f*) of tetranitromethane, as described in the Materials and Methods section. The line at the bottom of each gel is the position of the Bromophenol Blue marker dye.

Polyacrylamide-gel electrophoresis of Sephadex fractions

Tetranitromethane-treated enzyme was fractionated on Sephadex G-75 and samples of pooled fractions were analysed by sodium dodecyl sulphate/10% (w/v) polyacrylamide-gel electrophoresis after preincubation with sodium dodecyl sulphate and 2-mercaptoethanol for 2min at 100°C (Weber *et al.*, 1972). The results (Fig. 4) show a correlation between position of elution from Sephadex G-75 and apparent molecular weight after denaturation in a solution of sodium dodecyl sulphate. Thus the dimer fraction from the Sephadex column migrated mainly as a band of mol.wt. 63000, close to the value (58000) expected for dimer, and the multimer fraction migrated mainly as bands of molecular weight higher than that of bovine serum albumin.

Taken together with the results shown in Table 1, electrophoresis first confirms that the monomer was substantially free of material of higher molecular weight. Second, since the dimer fraction was essentially free of monomer after denaturation, it seems likely that the specific activity of this fraction (50% of normal) reflects the intrinsic activity of cross-linked dimer molecules. Retention of activity in dimer suggests that the tyrosine residues involved in cross-linkage are sufficiently far from the active site of at least one subunit of the dimer to permit substrate to approach the active site. Finally, the multimer fraction was essentially free of monomer; presumably it consisted of trimer and higher oligomers. The low but finite specific activity of this fraction could either be intrinsic to one or more of these oligomers or reflect contamination by dimer.

Ready intermolecular cross-linking rather than nitration possibly arises from steric or other environmental factors which favour reaction between tyrosine residues as an alternative to nitration. This is reasonable, because a phenoxide radical is probably a common intermediate in both nitration and cross-linking (Bruice *et al.*, 1968). In some proteins where reaction with tetranitromethane has led to selective nitration of tyrosine residues, with no detectable polymerization, e.g. ovalbumin, bovine plasma albumin, ribonuclease, lysozyme and concanavalin A (Doyle *et al.*, 1968), it is possible that the reactive groups are accessible to the reagent, but not sufficiently exposed to permit interaction between two residues in adjacent molecules.

Site of nitration

When tetranitromethane-treated penicillinase was digested with trypsin and chymotrypsin and the peptides were fractionated by gel filtration and high-voltage paper electrophoresis at pH6.5 (see the Materials and Methods section) a single dark acidic peptide (electrophoretic mobility relative to aspartate = 0.70) was detected under u.v. light. Its amino acid composition (Table 2) was essentially the same as that of the corresponding peptide (mobility = 0.73) shown by Ambler (1975) to be derived from residues 67–72. The corresponding peptide was isolated from the monomer fraction of tetranitromethane-treated enzyme. This peptide was identical with that isolated from unfractionated tetranitromethane-treated enzyme in elution volume from Sephadex G-25, in electrophoretic mobility at pH6.5, and in its position relative to other peptides on staining with fluorescamine. Its amino acid composition is shown in the final column of Table 2; it is evidently very similar to the previous analyses. Since the monomer fraction of the treated enzyme contained an average of 0.9 mol of nitrotyrosine/mol the ratio of nitrotyrosine to tyrosine recovered in this peptide represents 62% of the nitrotyrosine in the monomer fraction. The remaining 38% of nitrotyrosine was presumably accounted for by a low degree of substitution of more than one peptide; if it had been present on only one peptide it should have been detectable. It is concluded that nitration of tyrosine-72 has no effect on penicillinase activity.

The above results would be consistent with tyrosine-72 being remote from the active site, but since nitration does not markedly alter the stereo-

Table 2. *Amino acid composition of the major nitrotyrosine peptide from tetranitromethane-treated penicillinase*
Penicillinase (6mg) was reacted with equimolar tetranitromethane as described in the Materials and Methods section. Another sample (8.8mg) was reacted with a 2-fold molar excess of tetranitromethane before isolation of monomer material by gel filtration (for details, see the Materials and Methods section and Fig. 3). Peptides were isolated from both samples and subjected to amino acid analysis as described in the Materials and Methods section. In each case, amino acid analyses are normalized to Asp = 2.0. The theoretical composition for residues 67–72 is shown in parentheses.

	Amino acid composition of tetranitromethane-treated protein (number of residues/molecule)		
Amino acid	Results of Ambler (1975)	Unfractionated penicillinase	Nitrated monomer
Aspartate	2.0 (2)	2.0	2.0
Alanine	1.0 (1)	1.09	1.0
Valine	0.6 (1)	0.79	0.75
Isoleucine	0.7 (1)	0.68	0.57
Tyrosine	0.4 (1)	0.59	0.33
Nitrotyrosine	0.4	0.39	0.42
Glutamate	0.4	0.22	0.44
Glycine	0.12	0.22	0.49
Leucine	0.2	0.26	0.09
Serine	—	0.3	0.3
Threonine	—	<0.1	<0.1

chemistry of the tyrosine side chain, nitration of a residue in the active site might not affect activity. The following experiments were designed to test this possibility.

Reaction of monomer fraction with 1,5-*difluoro*-2,4-*dinitrobenzene*

When a nitrotyrosine residue is reduced the pK value of the amino group in the resulting aminotyrosine residue is much lower than those of other protein amino groups. The amino group may thus react as a nucleophile at pH5, whereas other protein amino groups are inert (Cuatrecasas *et al.*, 1969). The effect of attaching a bulky group to aminotyrosine residues (mainly at tyrosine-72) provides further evidence that this residue is not part of the active site. A bifunctional reagent was chosen with the aim of generating cross-linking between tyrosine and an adjacent nucleophile. Had activity been significantly decreased, either by attachment of the bulky group to tyrosine-72 or by subsequent cross-linking, this would have suggested that the residue was close to the active site.

To 42ml of a solution containing 20.6mg of monomer fraction of tetranitromethane-treated penicillinase (0.9mol of nitrotyrosine/mol of protein; specific activity 301 units/mg of protein) in $(NH_4)_2SO_4$/acetate buffer, pH5.0 (see the Materials and Methods section and legends to Fig. 3 and Table 1), was added a 200-fold molar excess of solid sodium dithionite. After incubation for 30min at room temperature the solution was dialysed for 48h against 2×2litres of $(NH_4)_2SO_4$/acetate buffer, pH5.0, at 4°C to remove excess sodium dithionite. The specific activity of the enzyme after dialysis was 303 units/mg of protein and A_{360} had fallen to zero, indicating complete reduction of nitrotyrosine residues to aminotyrosine. To the dialysed enzyme solution was added 100μl of a solution containing 1.7mg of 1,5-difluoro-2,4-dinitrobenzene in acetone, giving a 10-fold molar excess of the reagent. After incubation at room temperature for 1h, penicillinase was separated from excess reagent by passage through a column (2cm×38cm) of Sephadex G-25 (coarse grade), equilibrated and eluted with $(NH_4)_2SO_4$/acetate buffer, pH5.0. The excess reagent bound tightly to the column so that 36ml of solution was recovered containing protein at essentially unchanged concentration. The u.v. spectrum contained a new peak at 360nm. The specific activity at this stage was 316 units/mg of protein. After dialysis at 4°C for 4 days against 8×2litres of 0.45M-K_2SO_4/50mM-sodium acetate/27mM-acetic acid, pH5.0, the solution was adjusted to pH9.4 with 1M-NaOH and incubated for 48h at room temperature. Spectra at pH12 and pH3 (Cuatrecasas *et al.*, 1969) indicated that 2,4-dinitrophenolic groups were absent from the presumably cross-linked protein. Assuming that $\varepsilon_{360} = 1.65\times10^4$litre·mol^{-1}·cm^{-1} (Means & Feeney, 1971), then the average extent of binding of chromophore was 0.56mol/mol of enzyme. The protein solution (specific activity 398 units/mg of protein) was concentrated to 4ml by vacuum dialysis and passed through a column of Sephadex G-75 as described in the Materials and Methods section.

Monomer material accounted for 80% of the eluted protein.

Evidently, attachment of a bulky group to the enzyme, presumably mainly at tyrosine-72, and incubation under conditions which appear to have resulted in intramolecular cross-linking, did not decrease enzyme activity. This provides further evidence that tyrosine-72 is not close to the active site.

On the basis of this work tyrosine-72 cannot be regarded as simply more accessible to tetranitromethane than other tyrosine residues. However, selective nitration could arise from steric hindrance preventing tyrosine-72 from participation in intermolecular cross-linking. These results raise the possibility that selective nitration of the corresponding tyrosine residue in other β-lactamases may reflect similar steric hindrance rather than involvement in the active site. Since tyrosine-72 can be modified with no loss of enzyme activity, the residue may well be useful for attaching reporter groups or a heavy-atom substituent.

We thank Mrs. G. Burgham and Mr. R. B. Nicholson for help in preparing crude penicillinase. The work was supported by a research grant and a research studentship from the Science Research Council. We are especially grateful to Dr. R. P. Ambler for making available details of his sequence studies in advance of publication.

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