Affinity Labelling with a Deaminatively Generated Carbonium Ion

KINETICS AND STOICHEIOMETRY OF THE ALKYLATION OF METHIONINE-500 OF THE *lacZ* β -GALACTOSIDASE OF *ESCHERICHIA COLI* BY β -D-GALACTOPYRANOSYLMETHYL-*p*-NITROPHENYLTRIAZENE*

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(Received 20 December 1977)

1. β -D-Galactopyranosylmethyl-*p*-nitrophenyltriazenes are active-site-directed irreversible inhibitors of Mg^{2+} -bound and Mg^{2+} -free *lacZ* β -galactosidase from *Escherichia coli*. 2. The Mg^{2+} -enzyme binds the inhibitor more tightly but the complex then decomposes less rapidly than is the case with Mg^{2+} -free enzyme. 3. Loss of enzyme activity is a linear function of the fraction of enzyme protomers to which are attached β -D-galactopyranosyl[^{14}C]methyl residues: complete inactivation of fully active enzyme results in incorporation of 0.91 equivalent of carbohydrate label per enzyme protomer. 4. When the β -galactopyranosylmethyl cation is generated in the active site of Mg^{2+} -enzyme, it is captured essentially completely by the protein, but in the active site of Mg^{2+} -free enzyme it is only captured with an efficiency of 25%. 5. Labelled enzyme was carboxymethylated and digested with trypsin; acidic hydrolysis of the isolated tryptic peptide, and field-desorption mass spectrometry of the isolated radioactive derivative, showed it to be 2,5-dioxo-3-[2-(β -D-galactopyranosylmethylthio)ethyl]-1,6-trimethylenepiperazine. 6. This is considered to have arisen from labelling of the sulphur atom of a methionine residue adjacent to a proline residue. 7. The complete amino acid sequence of the molecule [Fowler & Zabin (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 1507–1510] enables the labelled methionine residue to be identified as either Met-421 or Met-500. 8. Sequence data [Fowler, Zabin, Sinnott & Smith (1978) *J. Biol. Chem.* in the press] show the site of attack to be Met-500.

The identification of amino acid residues in the active sites of liganding proteins, such as enzymes, by covalent attachment of reactive analogues of their substrates has become an increasingly common technique (Baker, 1967). There exists, however, a gap in the range of reactivities that can be incorporated into such an 'affinity label'. α -Halocarbonyl epoxy and α -diazoketone functional groups require the correct disposition of a significantly reactive group in the EI complex, whereas the photolytic generation of highly reactive carbenes and nitrenes can result in the attachment of the label to many sites.

It appeared that the carbonium ion species generated in deamination reactions might have intermediate reactivity. Soon after the inception of this work, the generation of such species in the active site of chymotrypsin, and their attachment thereto, was reported (White *et al.*, 1975). Because of our interest in the catalytic mechanism of the β -galacto-

sidase of *Escherichia coli*, we wished to generate cation (I) (Fig. 1) in the active site of *lacZ* enzyme, in the hope that the galactosylmethyl moiety would become attached to the group that donates a proton to the aglycone oxygen atom during the enzymic hydrolysis of galactosides.

Cation (I) could, in principle, be generated by the diazoalkane route, the nitrosoamide route and the triazene route (Fig. 1). It transpired, however, that *N*-(β -D-galactopyranosylmethyl)-*N*-nitrosobenzamide decomposed (probably by participation of the C-2 hydroxy group in the base-catalysed pathway for nitrosoamide decomposition) within the time of addition to solutions of pH 7. We also, like Brockhaus & Lehmann (1976), measured decreases of enzymic activity after stepwise addition to β -galactosidase of the methanolic solution obtained by treatment of *N*-nitroso-*N*-(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosylmethyl)acetamide with sodium methoxide, and probably containing β -D-galactopyranosyldiazomethane. However, in our hands the inhibition was associated with precipitation of protein and was too ill-defined for proper study. The

* The β -D-galactopyranosylmethyl residue is named the 2,6-anhydro-1-deoxy-D-glycero-L-manno-1-heptyl residue by IUPAC rules.

Fig. 1. Actual and potential routes to the active-site generation of the β -D-galactopyranosylmethyl cation

triazene route to generation of cation (I) in the enzyme active site proved successful, and a preliminary account of this has appeared (Sinnott & Smith, 1976). We now report full details of these experiments, further kinetic characterization of the system and the identity of the group labelled.

Alkylaryltriazenes, such as compound (II), exist as a mixture of tautomers, the one with the $-N=N-$ bond conjugated with the aromatic ring predominating (Isaacs & Rannala, 1974a; Iwamura *et al.*, 1976; Tronchet & Rachidzadeh, 1976). Both forms can

be detected, however, by ^1H and ^{13}C low-temperature n.m.r. spectroscopy, and the free-energy barrier to (IIa) $\rightleftharpoons$ (IIb)-like conversions has been estimated as 13.5 kcal/mol for methyl-*p*-tolyltriazene (Iwamura *et al.*, 1976) and about 14.5 and 16 kcal/mol for two carbohydrate *p*-nitrophenyltriazenes (Tronchet & Rachidzadeh, 1976). These values, which pertain to $[^2\text{H}]$ chloroform solutions, correspond to rates at 25°C of 10^1 – 10^3 s^{-1} . Tautomerizations will be faster in the more-polar protic solvent water, and, as rates of decomposition of the non-

covalent enzyme-triazene complexes are much slower than triazene tautomerizations, compound (II) can, for the present purposes, be regarded as one substance; in the case of methyl-*p*-tolyltriazene the barrier to tautomerization is in fact comparable with that to rotation about the N-N single bond (Iwamura *et al.*, 1976). Triazene isomers with a *cis*-azo linkage have never been detected.

The slow step in the decomposition of alkylaryl-triazenes (at least in organic solvents) is proton donation to the arene-proximal nitrogen atom of tautomer (IIa) (Isaacs & Rannala, 1974b). The fate of the protonated triazene then depends on the stability of the alkyl cation; if this is secondary, then fragmentation is synchronous, and significant quantities of *N*-alkylated aniline are formed by 'internal return', whereas if it is primary the alkyl diazonium ion lives long enough for the aniline to diffuse away from the encounter complex (Maskill *et al.*, 1965). The deamination of β -D-galactopyranosylmethylamine with HNO_2 , a deamination route similar to the three discussed above, gives substantial quantities of products arising from 1,2-shift of anomeric hydride ion (Coxon & Fletcher, 1964). The occurrence of this shift indicates that cation (I) is more stable than a simple primary cation; further stabilization from the lone pairs of electrons on the ring oxygen atom may also take place. The affinity labelling is therefore probably better described as being with a carbonium ion rather than with an aliphatic diazonium ion.

Experimental

Reagents

N-Ethylmorpholine. The laboratory-reagent-grade product (BDH, Poole, Dorset, U.K.) was redistilled over BaO and the fraction with b.p. 136–138°C was retained.

Buffer solutions. These were made by titration of the two components to the desired pH. Sodium phosphate (0.1M) buffer, pH 7.0, was made by admixture of 0.1M solutions of NaH_2PO_4 and Na_2HPO_4 , but α M-sodium EDTA buffers were obtained by adding concentrated NaOH to α M-EDTA, and likewise α M-*N*-ethylmorpholine/formic acid buffers were made by the addition of AnalaR formic acid to aq. redistilled α M-*N*-ethylmorpholine.

*3-*p*-Nitrophenyl-1-propyltriazene.* This was prepared by the method of White & Scherrer (1961). 1-Propylamine hydrochloride (4.8g) was dissolved in redistilled *NN*-dimethylformamide (15ml) and stirred with anhydrous Na_2CO_3 (7.5g) with cooling (by ice/salt). *p*-Nitrobenzenediazonium tetrafluoroborate (1g) in *NN*-dimethylformamide (5ml) was added dropwise over 10min. The solution was then allowed to warm to 0°C and was stirred for 1h.

Diethyl ether (20ml) was added to the mixture, and the solids were filtered off and washed with ether, which was then combined with the filtrate. This was then washed with water (3 × 50ml), dried (MgSO_4) and evaporated. The residue (0.5g; yield 57%) was recrystallized from light petroleum (b.p. 30–40°C) to give the *product*, m.p. 78–80°C (decomposition), λ_{max} (ethanol) 362nm (ϵ 22000) (Found C, 52.0; H, 5.6; N, 26.7; $\text{C}_9\text{H}_{12}\text{N}_4\text{O}_2$ requires C, 51.9; H, 5.8; N, 26.9%).

Unlabelled β -D-galactopyranosyl derivatives. 2,3,4,6-Tetra-*O*-acetyl- α -D-galactopyranosyl bromide, m.p. 84–85°C (literature value 84–85°C), was synthesized by the method of Lemieux (1963); β -D-galactopyranosylmethylamine, used without recrystallization (m.p. 180–183°C), was made by the method of Coxon & Fletcher (1964). Samples of methyl 1-thio- β -D-galactopyranoside (Sinnott, 1971) and *p*-nitrophenyl β -D-galactopyranoside (Sinnott & Souchart, 1973) have been described. β -D-Galactopyranosyl benzene was made by the action of phenyl Grignard reagent on 2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl bromide (Withers, 1977).

β -D-Galactopyranosyl [^{14}C]methylamine. KCN (0.586g) dissolved in water (5ml) was added to 50mCi of solid K^{14}CN (sp. radioactivity 60Ci/mol; The Radiochemical Centre, Amersham, Bucks., U.K.), giving a sp. radioactivity of ≈ 5.2 Ci/mol. The radioactive solution was transferred together with water washings (2ml) to a 50ml round-bottomed flask, and aq. 1.67M-mercuric perchlorate solution (3ml) was added. The resultant aqueous slurry was evaporated to dryness on a rotary evaporator, and dried *in vacuo* over P_2O_5 for 48h. A solution of 2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl bromide (2.1g) in dry nitromethane (10ml) was then added to the residue, and the resulting suspension was stirred for 24h at room temperature. The solids were then filtered off (glass-fibre filter papers) and washed with nitromethane (6ml). The combined washings and filtrate were evaporated, dried for 24h over P_2O_5 and extracted with warm dichloromethane (3 × 13ml). The dichloromethane extract was filtered (glass-fibre filter papers), extracted three times with an equal volume of aq. 1M-KBr solution, then with water, dried with anhydrous MgSO_4 and evaporated.

The resultant pale syrup was dried *in vacuo* over P_2O_5 for 24h, and then reduced, without further purification, with LiAlH_4 , as described by Coxon & Fletcher (1964), to give β -D-galactopyranosylmethylamine, m.p. 180–185°C (yield 0.236g, 24%; sp. radioactivity 4.1Ci/mol). The combined mother liquors and washings contained a further 3.5mCi of impure galactosylmethylamine.

*β -D-Galactopyranosyl [^{14}C]methyl-*p*-nitrophenyl-triazene.* Crystalline β -D-galactopyranosyl [^{14}C]-

methylamine (0.244 mmol) was dissolved in ice-cold water (1 ml), *p*-nitrobenzenediazonium tetrafluoroborate (27 mg; 0.122 mmol) was added, and the whole was stirred for 30 min at 0°C, after which time the solution did not give a red precipitate with alkaline 2-naphthol. Ice-cold water (2 ml) was added and the solids were filtered off. To remove diazonium hydroxide the filtrate was extracted three times with its own volume of ice-cold redistilled ether, which had been saturated with water. Triazene was then extracted into butan-1-ol (3-fold extraction with $\frac{1}{3}$ vol. of ice-cold butan-1-ol), and the combined butanol extracts were evaporated to dryness at room temperature under trolley-pump vacuum (0.01 mmHg). The residue was then dissolved in ice-cold water (1 ml) and used immediately without further purification. Triazene concentration was estimated from its u.v. absorbance (see the Results and Discussion section). On this basis the stock solution was typically 25–30 mM, corresponding to a yield of 20–25 %.

Unlabelled triazene was made by the same procedure.

Kinetics of interaction of β -galactosidase and triazene

β -D-Galactosidase (EC 3.2.1.23). Initial experiments were carried out with commercial preparations of the enzyme. Crystalline enzyme, free of bovine serum albumin, from *E. coli* ML 308 (lot no. 7423216) was purchased as a suspension (5 mg/ml) in 2.2 M-(NH₄)₂SO₄ solution from the Boehringer Corporation, London W.5, U.K. A suspension (10 mg/ml) of crystalline enzyme (lot no. BGC N5G359) in (NH₄)₂SO₄ solution was purchased from Worthington Biochemical Corporation, Freehold, NJ, U.S.A.

An enzyme of significantly higher specific activity was purified from lysates of an episome-containing constitutive strain (A324-5) of *E. coli* K-12, merodiploid with respect to the *lac* operon (Fowler, 1972), essentially as described by Craven *et al.* (1965). Starting from sedimented bacterial cells (130 g wet weight) this procedure gave 840 mg of substantially pure enzyme in 49.5 % yield, and resulted in an 8.1-fold purification over crude lysate. When assayed under the conditions stated below, the specific activity of this preparation (38.3 μ mol/min per mg of protein) was, within experimental error, the same as that of a sample kindly provided by Dr. A. V. Fowler (40.6 μ mol/min per mg of protein), which had been used for the elucidation of its complete amino acid sequence. A subunit mol.wt. of 120 000 is assumed for this tetrameric enzyme.

Spectrophotometric measurements. These were made on a Unicam SP. 1800 recording-spectrophotometer system, fitted with a cell-block through which water maintained at $25.0 \pm 0.1^\circ\text{C}$ was passed by a Tecam

Tempunit, or on the digital-output version of the same instrument (SP. 1700), in which case the thermostatic pump was a Julabo Paratherm U4 model.

Spontaneous and enzyme-catalysed triazene decomposition. This was followed by the decrease in A_{362} . First-order rate constants for the spontaneous hydrolysis were calculated by the Guggenheim (1926) method, but for enzyme-catalysed reactions experimental infinity readings were used.

Enzyme assays. Production of *p*-nitrophenol from *p*-nitrophenyl β -D-galactopyranoside (0.3 mM; 10 K_m) (Sinnott & Souchard, 1973) in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-MgCl₂, was monitored spectrophotometrically at 400 nm and 25°C. Plastic cells (1 cm path-length; Pye-Unicam, no. 681243) containing substrate solution (3 ml) were used.

Measurement of radioactivity. ¹⁴C radioactivity was counted in an ICN Tracerlab Spectromatic liquid-scintillation counter. Counting efficiency was determined by the external-standard procedure with a standard quench-corrective curve. To the samples was added scintillation 'cocktail' (10 ml) consisting of 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole (5 g/l; Koch-Light, Colnbrook, Bucks., U.K.) in 2-methoxyethanol/toluene (1:4, v/v).

Kinetics of β -galactosidase inactivation. β -Galactosidase (0.5 mg/ml) in either 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.5 mM-MgCl₂, or Mg²⁺-free β -galactosidase (Tenu *et al.*, 1972) in 0.05 M- or 0.1 M-sodium EDTA buffer, pH 7.0, was inactivated at various triazene concentrations in a thermostatically controlled water bath at $25 \pm 0.1^\circ\text{C}$. Portions (5 μ l) were removed at appropriate intervals and assayed. First-order rate constants were calculated by linear least-squares treatment of log (activity)-time plots. In experiments in which [¹⁴C]triazene was used, the rate of radioactive labelling of the enzyme was measured simultaneously by removing portions (0.1 ml) at similar time periods. Each portion was immediately pipetted into ice-cold aq. 10 % (w/v) trichloroacetic acid (1 ml) followed by 1.0 % (w/v) ovalbumin solution (0.05 ml) as co-precipitant, and left for 1 h at 0°C to complete precipitation of enzyme. The protein was filtered off, through HAWP 02500 filters [Millipore (U.K.), London, U.K.] washed with ice-cold aq. 5 % (w/v) trichloroacetic acid solution (5 $\times$ 10 ml), dried in air and counted for radioactivity.

Determination of 'weighted-mean' K_i for triazene-decomposition products. A 7.54 mM solution of β -D-galactopyranosyl-*p*-nitrophenyltriazene in water was allowed to decompose at room temperature for 24 h, and then evaporate to dryness. The residue was taken up in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1 mM-MgCl₂, and used as stock solution for the K_i determination, which was against *o*-nitrophenyl β -D-galactopyranoside ($K_m = 0.1$ mM;

Sinnott & Souchard, 1973), liberation of *o*-nitrophenol being followed spectrometrically at 400nm. The initial rates (25 at each of 55, 110, 165, 220 and 275 μ M concentrations of substrate and 0, 125, 251, 377 and 502 μ M concentrations of parent triazene) were measured with 2mm-path-length cells because of the high absorbance of inhibitor. These initial rates were fitted directly to the expression for competitive inhibition, with a library program for a Hewlett-Packard 9100B calculator.

T.l.c. analysis [on silica gel G, with butan-1-ol/acetic acid/water (4:1:1, by vol.) as eluent] of the decomposition products of this triazene indicated the main chromophoric product to be *p*-nitroaniline.

Protein concentration. This was determined spectrophotometrically assuming $A_{280}^{1\%} = 20.9$ (Craven *et al.*, 1965) or by the biuret procedure (Gornall *et al.*, 1949) with crystalline bovine serum albumin as standard. The two methods agreed within 5%.

Product and manipulation of labelled protein

All reagents were AnalaR grade or better, except where stated.

Bulk labelling of enzyme. A solution of β -galactosidase (5mg/ml) previously dialysed against 0.1M-sodium EDTA buffer, pH7.0, was reacted with a 30-fold molar excess of [14 C]triazene (sp. radioactivity 4.1 or 1 Ci/mol) overnight at room temperature. The inactivated enzyme, possessing 2.5% of its initial activity, was dialysed against 10mM- NH_4HCO_3 (3×4 litres) at 4°C, and then passed through a column (3cm $\times$ 45cm) of Sephadex G-25 equilibrated with the same buffer. The excluded 14 C-labelled protein (of sp. radioactivity 0.907 Ci/mol in experiments carried out with enzyme from the merodiploid strain and [14 C]triazene of sp. radioactivity 1 Ci/mol) solution was shell-frozen and freeze-dried.

A similar procedure with Mg^{2+} -enzyme in 0.1M-sodium phosphate buffer, pH7.0, containing 1.0mM- MgCl_2 , afforded radioactive protein of sp. radioactivity 0.905 Ci/mol.

Reduction and carboxymethylation of 14 C-labelled β -galactosidase. The radioactively labelled protein was reduced and carboxymethylated essentially as described by Craven *et al.* (1965). Freeze-dried radioactively labelled β -galactosidase was dissolved in 8M-urea (previously deionized by passage through a mixed-bed ion-exchange column of Amberlite IR-120 and IRA-410) containing 0.1M-2-mercaptoethanol and buffered with 0.1M- NH_4HCO_3 , to a final concentration of 5–10mg of protein/ml. This mixture was then incubated at 45°C for 4h, and then iodoacetic acid, which had been twice recrystallized from light petroleum (b.p. 40–60°C), was introduced in a 5-fold molar excess over the 2-mercaptoethanol,

the pH being kept constant by the addition of 6M-NaOH. The reaction was stopped after 15–20min by the addition of an excess of 2-mercaptoethanol. The product was exhaustively dialysed against 0.2M- NH_4HCO_3 , and this solution was used directly for trypsin digestion.

Trypsin digestion. By the procedure of Steers *et al.* (1965), the dialysed carboxymethylated and radioactively labelled solution was incubated with bovine pancreatic trypsin (type XI; Sigma Chemical Co., London S.W.6, U.K.) at 37°C for 5h. A protein/trypsin ratio of 50:1 by weight was used. On completion of the digestion, the reaction mixture was immediately freeze-dried.

Radioactive-peptide 'mapping'. The 'mapping' of tryptic hydrolysates was carried out by the procedure of Steers *et al.* (1965). Freeze-dried radioactive peptide mixture (3mg) dissolved in dilute aq. NH_3 containing Phenol Red as visible marker (40–50 μ l) was spotted on a 57cm sheet of Whatman 3MM paper at a point 10cm from one end. The paper was then eluted downwards with the organic phase butan-1-ol/acetic acid/water (4:1:5, by vol.) as chromatographic solvent. On completion of the chromatography (24h) the paper was air-dried and the position of the Phenol Red spot was marked. The chromatogram was then stitched on to a fresh 57cm sheet of Whatman 3MM paper, 10cm from the anode end, and was subjected to electrophoresis (1h at 3000 V) at pH3.5 [electrolyte 5% (v/v) acetic acid, 0.5% (v/v) pyridine in water] under toluene. After thorough drying of the electrophoretogram 14 C-labelled peptides were located by radioautography with Kodak X-ray safety film (F11 M-5) and an exposure time of 80h. Non-radioactive peptides were detected with cadmium/ninhydrin (Heilmann *et al.*, 1957).

Attempted removal of label with cyanogen bromide. Freeze-dried reduced and carboxymethylated protein approx. 60mg) was dissolved in aq. 70% formic acid (10ml) and a solution of CNBr (0.58ml; 98mg/ml) in the same solvent added. After reaction overnight at room temperature, the reaction mixture was diluted 10-fold with water and freeze-dried. After dissolution in aq. 30% (v/v) acetic acid (10ml) to which a few drops of formic acid had been added, the products were chromatographed on a Sephadex G-100 column (2.5cm $\times$ 80cm) with aq. 30% (v/v) acetic acid as eluent at a flow rate of 30ml/h; 20% of the radioactivity was in the completely included fraction.

In a further experiment, the tryptic peptides (3mg) in aq. 70% (v/v) formic acid (0.5ml) were allowed to react with CNBr (20mg) for 48h. After work-up as before, the sample was subjected to paper electrophoresis (3000 V for 1h) at pH6.5 (pyridine/acetic acid as electrolyte) and 40% of the applied radio-

activity was found in a tight neutral band that had moved towards the cathode by electroendosmosis.

Isolation of labelled tryptic peptide. Freeze-dried tryptic peptide (55mg; labelled with triazene of sp. radioactivity 4.1 Ci/mol) was dissolved in dilute aq. ammonia (4.0ml) and chromatographed on a Sephadex G-25 (fine) column (1.5cm × 90cm) with 0.2M-NH₄HCO₃ as eluent at a flow rate of 43ml/h. Fractions 24–28 were pooled (see Fig. 7), diluted with 4vol. of water and freeze-dried. The freeze-dried powder was taken up in 10mM-N-ethylmorpholine/formic acid buffer, pH 7.0, and applied to a column (2.5cm × 20cm) of DEAE-cellulose (Whatman DE-52, prepared by the manufacturer's instructions) equilibrated with this buffer, and 1 column volume of 10mM buffer passed through. A linear concentration gradient (10–500mM) was then applied, at a flow rate of 30ml/h with a total throughput of 1 litre. Fractions 34–36 (see Fig. 8), accounting for 68% of the applied radioactivity, were pooled and freeze-dried.

Amino acid composition of peptides. Purified peptides were hydrolysed in constant-boiling HCl in evacuated, sealed ampoules at 105–110°C for 16–48 h. The ampoules were cooled, broken open, and the contents evaporated to dryness under filter-pump vacuum (bath temperature 40–50°C). The amino acid analysis of the residue was carried out on a Rank Hilger Chromaspek T180 analyser.

Isolation of galactosylmethylated derivatives. The residue from the hydrolysis of approx. 0.1 μmol of radioactively labelled tryptic peptide was taken up in aq. 1% (v/v) acetic acid and applied to a column (80cm × 1.7cm) of Sephadex G-10 equilibrated with the same solvent. Elution at a rate of 30ml/h with this solvent gave one main radioactive peak. The elution volume of tyrosine was determined under identical conditions.

The fractions containing the main radioactive derivative were freeze-dried, and the contents of the flask were washed out with water into a small vial, which was then dried over solid NaOH *in vacuo*.

Mass spectrometry. Low-resolution field-desorption mass spectra were recorded on a Varian mass spectrometer equipped with a combined electron impact/field ionization/field-desorption source. The source temperature was 80°C and accelerating voltage 3000 V. Solutions of the sample in ethanol or chloroform were evaporated on to the field-desorption probe and inserted directly into the spectrometer. Low-resolution electron-impact mass spectra were recorded with a Finnigan GC/EI-CI spectrometer system fitted with a continuous metal-band delivery system for insertion of sample.

Results and Discussion

After preliminary work had shown that solutions containing β-D-galactopyranosylmethyl-*p*-chloro-

phenyltriazene irreversibly inhibited β-galactosidase, that this inhibition was overcome by known competitive inhibitors of the enzyme, but that the lability of alkylaryltriazenes made the acquisition of clear-cut kinetic data difficult, semi-quantitative 'range-finding' experiments were performed. These indicated that: (i) electron-withdrawing substituents in the aryl moiety of the triazene slowed down the spontaneous decomposition; in fact the rates of decomposition of aryl-substituted arylbenzyltriazenes at pH 7.0 and 25°C are correlated by Hammett σ values with a ρ value of –1.9 (C. C. Jones & M. L. Sinnott, unpublished work); (ii) the spontaneous decomposition of the *p*-nitro derivative was not greatly influenced by pH above the value of 7, but on further lowering of the pH accelerated sharply; (iii) the rates of spontaneous decomposition and enzyme inactivation were comparably temperature-sensitive.

Therefore all further experiments were performed with the *p*-nitrophenyltriazene at 25°C.

β-D-Galactopyranosylmethyl-*p*-nitrophenyltriazene successfully resisted crystallization. Its concentration was therefore estimated on the assumption that its u.v. absorption in ethanol was the same as that of *p*-nitrophenyl-*n*-propyltriazene, which had been more thoroughly characterized. The error introduced into binding-constant values by this procedure is unlikely to exceed 5–10%, since both triazene (II) and the model compound have the same chromophore and primary alkyl groups. Even the change in the alkyl group R of R–NH–N=N–*p*-C₆H₄NO₂ from primary to secondary (from R = CH₃[CH₂]₇– to R = CH₃[CH₂]₂–CH–[CH₂]₃CH₃) alters $A_{\max}$ by only 10% (Southam, 1965). The splitting pattern of the resonances of the aromatic protons of triazene (II) (in ²H₂O) was identical with that from 3-(*p*-nitrophenyl)-1-propyltriazene (in [²H]chloroform).

Kinetics of deactivation

Typical time courses of the inactivation of Mg²⁺- and Mg²⁺-free β-galactosidase by an excess of triazene (II) are displayed in Fig. 2. The loss of enzyme activity of Mg²⁺-free enzyme is accurately first-order. The linearity of the Eadie plot in Fig. 3(b) shows that under these conditions of a large excess of triazene, the deactivation is described by eqn. (1):

where E* is enzymically inactive and Tr represents triazene. The dissociation constant, K , of the covalent complex with Mg²⁺-free enzyme derived from Fig. 3(b) is 0.48mM and $k_{\text{deact.}}$ is $9.8 \times 10^{-3} \text{ s}^{-1}$. These values, which pertain to 0.1M-sodium EDTA buffer at pH 7.0, were obtained with ¹⁴C-labelled triazene, and differ somewhat from the values obtained in 0.05M-sodium phosphate buffers (0.21mM and

Fig. 2. Variation of β -D-galactosidase activity with time in the presence of β -D-galactopyranosylmethyl-p-nitrophenyl-triazene at pH7.0 and 25°C
 (a) Sodium phosphate buffer (0.05M), 1mM-MgCl₂, [triazene] = 6.7mM, [E]₀ = 0.035mg/ml. (b) Sodium EDTA buffer (0.1M), [triazene] = 85 μ M, [E]₀ = 0.49mg/ml.

$5.7 \times 10^{-3} \text{ s}^{-1}$ respectively) and reported in a previous communication (Sinnott & Smith, 1976).

The inactivation of Mg^{2+} -enzyme is much slower than that of Mg^{2+} -free enzyme, and so the spontaneous decomposition of the triazene is correspondingly more important. This spontaneous decomposition is also catalysed by the medium used for inactivation of Mg^{2+} -enzyme (k is $7.9 \times 10^{-5} \text{ s}^{-1}$ in this medium and $3.4 \times 10^{-5} \text{ s}^{-1}$ in the medium used for inactivation of Mg^{2+} -free enzyme), but this accelerated decomposition is still insufficient to rationalize the curvature of the plots in Fig. 2(a). That this curvature is more pronounced at lower concentrations indicates that some transformation of the reagent, rather than enzyme inhomogeneity or co-operativity amongst the four subunits of the enzyme, is responsible. In fact solutions of triazene that have been allowed to decompose completely are strong (competitive) inhibitors of the enzyme, with

Fig. 3. Dependence of observed first-order rate constants at 25°C for decay of enzyme activity on concentration of β -D-galactopyranosylmethyl-p-nitrophenyltriazene
 (a) Mg^{2+} -enzyme, 0.05M-sodium phosphate buffer, pH7.0, 1mM-MgCl₂. Symbols: $\blacktriangle$, no competitive inhibitor; Δ , in the presence of 9mM-methyl 1-thio- β -D-galactopyranoside. (b) Mg^{2+} -free enzyme, 0.1M-sodium EDTA buffer, pH7.0, [E]₀ = 0.49mg/ml.

an apparent K_i of 0.23 ± 0.04 mM. The spontaneous decomposition of the triazene generates a protecting agent. The first-order rate constants displayed in Fig. 3(a) are therefore derived from the initial linear portions of the semi-logarithmic activity plots. These data indicate that the enzyme is protected against inactivation by triazene (II) by the known competitive inhibitor methyl 1-thio- β -D-galactopyranoside. In the absence of methyl thiogalactoside they conform to eqn. (1) with $K = 73 \pm 11 \mu\text{M}$, $k_{\text{deact.}} = 4.0 \times 10^{-4} \text{ s}^{-1}$; in the presence of 9 mM-inhibitor (III), K becomes 0.54 mM, but $k_{\text{deact.}}$ remains constant ($3.7 \times 10^{-4} \text{ s}^{-1}$). The K_i value for methyl 1-thio- β -D-galactopyranoside calculated from its inhibition of enzyme inactivation is 1.4 mM; that calculated from its inhibition of the catalytic activity of the enzyme is 1.8 mM (Sinnott, 1971).

The inhibition is therefore active-site-directed. This conclusion was reinforced by replacing β -D-galactopyranosylmethylamine by 1-propylamine in the triazene synthesis but proceeding otherwise as normal; rates of inactivation of <5% of those previously observed were obtained.

Fig. 4. Dependence of rate of inactivation of Mg^{2+} -free enzyme on pH at 25°C

[Triazene] = 1.96 mM, $[E]_0 = 0.035$ mg/ml, buffer solutions were 0.05 M-EDTA adjusted with concentrated NaOH. The line represents a theoretical maximal inactivation rate of $8 \times 10^{-3} \text{ s}^{-1}$ governed by a pK_a of 7.5.

The rate of loss of enzymic activity of Mg^{2+} -free enzyme in 1.96 mM-triazene (II) is shown as a function of pH in Fig. 4. At pH 7 the enzyme is 78% saturated with triazene. The solid line is that described by the equation:

$$k_{\text{app.}} = 8 \times 10^{-3} / \left(1 + \frac{3 \times 10^{-3}}{[\text{H}^+]} \right) \text{ s}^{-1}$$

However, this curve has only operational significance, since as yet we do not know whether the observed variation is due to a change in the rate of protonation of the triazene, a change in the efficiency of capture of the carbonium ion (see under 'Stoichiometry of inhibitors') or a very large change in the binding constant.

Stoichiometry of inhibition

Fig. 5 shows mol of β -D-galactopyranosyl ^{14}C -methyl residue incorporated per mol of β -galactosidase protomer, plotted against residual enzymic activity. That essentially the same line is defined by five different triazene concentrations, ranging over an order of magnitude, indicates that non-specific incorporation is not significant. Such behaviour is expected from a 'hot carbonium ion'-based affinity label. The actual alkylating agent, the carbonium ion, has a lifetime of the order of nanoseconds or

Fig. 5. Correlation between galactosyl ^{14}C -methyl residues incorporated into protein and enzymic activity remaining

Mg^{2+} -free β -galactosidase (Boehringer; 0.49 mg/ml) in 0.1 M-sodium EDTA buffer at 25°C . The data pertain to the following triazene concentrations: $\circ$, 1.09 mM; $\blacksquare$, 0.710 mM; $\square$, 0.419 mM; $\triangle$, 0.199 mM; $\bullet$, 0.085 mM incubated with enzyme for various time periods under the conditions of Fig. 2(b), as described in the Experimental section.

less, and is highly reactive. It is consequently very non-specific and, if generated anywhere apart from the active site, will react with water or buffer components. The whole of the specificity of the reagent is accounted for by the binding specificity of the galactosylmethyl moiety for the enzyme. Conventional α -halocarbonyl-based affinity labels do not have this advantage.

The stoichiometry of reaction apparently shown in Fig. 5 (0.64 mol of label incorporated per mol of enzyme protomer) is a consequence of our use of commercial (Boehringer) β -galactosidase. Enzyme concentrations were estimated assuming pure active protein and $A_{280}^{1\text{mg/ml}}$ of 2.1. With enzyme isolated from merodiploid A-324/5 strain a stoichiometry of 0.91:1 was observed on the same assumptions.

Therefore, for every protomer of β -galactosidase inhibited, an equivalent of β -D-galactopyranosylmethyl is incorporated into the protein. Further, there is no sign in Fig. 5 of any co-operativity between sites.

Since cation (I) could react with water molecules in the active site, it does not necessarily follow that for every protomer of β -galactosidase inactivated only one molecule of triazene is decomposed. Even though triazene (II) was being decomposed in the enzyme active site by the operation of groups on the enzyme, cation (I) could still react with water or buffer components in the active site. Under these conditions the enzyme would be catalysing the decomposition of triazene. The only way that this catalysis could be detected is by following the rate of decomposition of triazene in the presence of comparable molar concentrations of enzyme, and observing an enhanced rate of decomposition.

Fig. 6 shows the results of such experiments. The kinetic system is described by eqns. (2). The differential equations describing such a reaction scheme cannot be solved explicitly, so the time course of triazene decomposition was computed iteratively, by using values of K and $k_{\text{deact.}}$ obtained from the experiments in which loss of enzymic activity was followed.

Fig. 6. Efficiency of capture of the β -D-galactopyranosylmethyl cation by Mg^{2+} -bound and Mg^{2+} -free β -galactosidase at 25°C : time course of triazene decomposition with $[E]_0 \approx [Tr]$

(a) Mg^{2+} -free enzyme ($4.1 \mu\text{M}$) and triazene ($45 \mu\text{M}$) in 0.1 M -sodium EDTA buffer, pH 7.0, in the absence ($\blacksquare$) and presence ($\square$) of β -D-galactopyranosylbenzene (50 mM ; $7 K_i$). The continuous lines are computed with $10^2 k$ (s^{-1}) = 3.92 (1); 3.5 (2); 3 (3); 2.8 (4 and 5). For other details of eqns. (2) see the text. (b) Mg^{2+} -enzyme ($4.1 \mu\text{M}$) and triazene ($37.5 \mu\text{M}$) in 0.1 M -sodium phosphate buffer, pH 7.0, containing 1 mM - MgCl_2 . The continuous lines are computed with $10^4 k$ (s^{-1}) = 4 (1); 2 (2); 1 (3). For further details see the text.

For Mg^{2+} -free enzyme, a good fit in the absence of the competitive inhibitor, galactosylbenzene, is obtained with k adjusted to $2.8 \times 10^{-2} \text{ s}^{-1}$. With this value, and a value of K_i for galactosylbenzene of 7 mM (Withers, 1977), the time course in the presence of this competitive inhibitor is predicted accurately. Therefore the catalysis of triazene decomposition without concomitant loss of enzyme activity that has been observed is an active-site phenomenon. The cation (I) is in fact being captured by the protein of β -galactosidase with only 25% efficiency, even though it is being generated in the active site. The remaining 75% is presumably reacting with water or

buffer components. Triazene (II) is therefore a suicide substrate of Mg^{2+} -free β -galactosidase, being decomposed in the E·Tr complex some 800 times faster than in free solution.

Since the enzyme-catalysed decomposition of triazene (II) was followed spectrophotometrically, the above conclusions are dependent upon the enzyme-catalysed reaction giving materials of comparable absorbance to the products of the spontaneous decomposition. Since the predominant chromophoric product of this latter process is *p*-nitroaniline (as shown by t.l.c.), and since triazene decomposition is associated with a substantial decrease in absorbance, any difference in the product ranges of the spontaneous and enzyme-catalysed processes is unlikely to affect the results. In fact, if loss of enzyme activity is followed as a function of time with $[E] \approx [Tr]$, the curve-fitting procedure gives a capture efficiency of 18%. This value is not dependent on assumed absorption coefficients of the products, but will be artificially low because of triazene-decomposition products already present at zero time.

For Mg^{2+} -enzyme Fig. 6(b) indicates that the catalytic process is less important than the deactivation process. The computed time course is comparatively insensitive to the value chosen for k , and we cannot really distinguish between 80% capture and 100% capture. Therefore decomposition of the triazene in the complex with Mg^{2+} -enzyme is only 4–5-times faster than in free solution.

This difference in kinetic behaviour of Mg^{2+} -bound and Mg^{2+} -free enzyme is almost certainly related to triazene decomposition being initiated by

Fig. 7. Chromatography on Sephadex G-25 of the tryptic digest of reduced carboxymethylated β -D-galactopyranosyl- $[^{14}C]$ methyl-labelled Mg^{2+} -free β -galactosidase. ■—■, Radioactivity. ···, Percentage transmission at 280 nm with a path-length of 1 cm. NH_4HCO_3 (0.2M) was passed through a bed (90 cm $\times$ 1.5 cm) of stationary phase at a rate of 43 ml/h and 6 ml fractions were collected.

Fig. 8. DEAE-cellulose chromatogram of the radioactive fraction from Sephadex G-25 chromatography. A linear gradient (10–500 mm) of *N*-ethylmorpholine/formic acid buffer, pH 7.0, was applied from fraction 20 onwards, at a flow rate of 30 ml/h, to a bed (20 cm $\times$ 5 cm) of ion-exchanger. Fractions (6 ml) were collected. ■—■, Radioactivity. ···, Percentage transmission at 280 nm with a 1 cm path-length. TA and TB indicate the positions of peptides TA and TB respectively.

proton donation to the distal nitrogen atom of the glycone. In Mg^{2+} -free enzyme the groups normally co-ordinated to Mg^{2+} , as well as the acid-catalytic group, are available for proton donation, since there is competition between protons and Mg^{2+} (Tenu *et al.*, 1971).

Identification of labelled residue

The strategy adopted was first to isolate an active-site peptide and then hydrolyse the peptide, rather than to attempt to isolate a single modified amino acid of the 1021 residues present in the molecule directly (Fowler & Zabin, 1977). The amino acid sequence of the tryptic peptide, and its location in the overall sequence of the molecule, is known (Fowler *et al.*, 1978).

Peptide 'mapping' of the trypsin digest of carboxymethylated labelled Mg^{2+} -enzyme and Mg^{2+} -free enzyme showed that radioactivity was incorporated into the same peptide in both cases (result not shown).

Fig. 7 shows the elution profile resulting from chromatography of the tryptic digest of carboxymethylated labelled Mg^{2+} -free enzyme on Sephadex G-25; Fig. 8 shows the chromatogram resulting from re-chromatography of the radioactive peak (fractions 24–28) on DEAE-cellulose. Minor peptide TB is derived from peptide TA by a contaminating chymotrypsin activity in the trypsin preparation (Fowler *et al.*, 1978). Peptide TA was then hydrolysed in constant-boiling HCl at 105–110°C for 14 h, and

subjected to chromatography on a Sephadex G-10 column previously calibrated with tyrosine (Fig. 9).

Attempts to subject the modified amino acid to gas chromatography as its *NO*-bis(trifluoroacetyl) methyl ester (Zanetta *et al.*, 1972) failed. The derivative was then subjected to field-desorption mass spectrometry. This technique for obtaining the mass spectra of involatile materials uses the very high voltage gradient at a sharp point to ionize the compound directly from the surface. Fragmentation is minimized, but the molecular ion can be augmented by hydrogen atoms or even alkali-metal atoms. For a recent critical review see Derrick (1977). Table 1 shows the spectrum produced from the highest-molecular-weight (and most plentiful) radioactively labelled derivative. The clean pattern of peaks indicates that the modified amino acid is substantially pure. Assignment of m/e 413 as $(M + Na)^+$ was confirmed by re-running the spectrum with chloroform as application solvent, when it disappeared. This pattern of peaks was similar to that produced from β -D-galactopyranosylmethylamine: the base peak [m/e 194 for $(M + H)^+$] was accompanied by

Fig. 9. Chromatography of the acid hydrolysate of peptide TA on Sephadex G-10

Acetic acid (1%) was passed through a bed (80cm $\times$ 1.7cm) of gel at a rate of 30ml/h. Fractions (6ml) were collected. The arrow refers to the elution position of tyrosine (mol.wt. 181).

parent ion m/e 193 (4%) and $(M + H)^+ {}^{13}\text{C}$ satellite (8%). The intensity of the m/e 393 signal is in accord with the 6% enrichment in ${}^{14}\text{C}$ calculated from radioactivity measurements.

A molecular ion of 390 is too heavy to have arisen from any single glycosylmethylated amino acid. We therefore suspected that the glycosylmethylation had stabilized a peptide linkage to hydrolysis, and accordingly re-hydrolysed the derivative for 48h. Amino acid analysis of the hydrolysate showed that 1mol of proline had been liberated per mol of label (estimated on the basis of its radioactivity). No other amino acid was present in stoichiometric amounts, although a number of degradation products were apparent, the major one appearing before methionine and corresponding to roughly 0.6mol per mol of label (assuming an 'average' ninhydrin calibration factor). An unknown radioactive derivative was also produced.

Therefore the isolated derivative is a proline-containing dipeptide or derived therefrom. However, none of the residues next to proline in the amino acid sequence (Fowler & Zabin, 1977) will, when galactosylmethylated, give a mol.wt. of 390. A parent ion of 390 is, however, compatible with (prolyl)-galactosylmethylated homocysteine if cyclization to a dioxopiperazine has taken place. Such cyclization takes place especially readily with prolyl amino acids, occurring even in aqueous solution (Sammes, 1975).

β -Galactosidase does not contain any homocysteine, although an *S*-galactosylhomocysteine residue could have arisen from de-*S*-methylation of an *S*-(galactosylmethyl)methionine residue. Decomposition of such a derivative by nucleophilic attack at the least-sterically-hindered site, the methyl group, is likely during manipulation of the labelled protein. Thus carboxymethylation was performed in the presence of (highly nucleophilic) 2-mercaptoethanol. The conclusion that cation (I) has become attached to the sulphur atom of a methionine residue, and the resulting sulphonium salt has lost a methyl group, is confirmed by the effect of attempted CNBr cleavage of labelled protein. Under the conditions

Table 1. Field-desorption mass spectrometry of the highest molecular-weight radioactively labelled derivative

m/e	Intensity (%)	Assignment
112	14.02	?
120	7.50	?
390	53.86	M^+
391	100.00	$[{}^{13}\text{C}]M^+$, $(M + H)^+$
392	40.06	$[{}^{14}\text{C}]M^+$, $[{}^{34}\text{S}]M^+$, $([{}^{13}\text{C}]M + H)^+$, $(M + 2H)^+$
393	9.37	$([{}^{14}\text{C}]M + H)^+$, $([{}^{34}\text{S}]M + H)^+$, $([{}^{13}\text{C}]M + 2H)^+$
413	14.47	$(M + Na)^+$

Fig. 10. Molecular events leading to the formation of the isolated derivative

recommended by Steers *et al.* (1965), 20% of the radioactivity is lost as a low-molecular-weight fragment; under forcing conditions this increases to 40%. It is to be expected that electrophilic attack by CNBr on an *S*-(galactosylmethyl)homocysteine residue will be less ready than on methionine for both steric and electronic reasons.

Methionine-421 and methionine-500 are next to proline residues in the amino acid sequences of the protein. We therefore ascribe structure (III) to the isolated derivative. The molecular events leading from active-site generation of cation (I) to isolation of structure (III) are shown in Fig. 10.

Identification of Met-500 as the target attacked by our affinity label was first made on the basis of sequence studies (Fowler *et al.*, 1978). However, the serendipitous stability of the prolyl-[*S*-(galactosylmethyl)]homocysteine peptide bond enables us to identify the target methionine as either Met-421 or Met-500 purely on the evidence given above.

Naider *et al.* (1972) described alkylation of a catalytically inessential methionine residue in *lacZ* β -galactosidase *N*-(bromoacetyl)- β -D-galactopyranosylamine. This was almost certainly also Met-500, although with this derivative nucleophilic attack was on the resulting sulphonium salt at the activated position and to the carbonyl function proceeded preferentially, with concomitant loss of label. However these authors worked with Mg^{2+} -enzyme. We have shown that the same tryptic peptide is labelled, irrespective of whether Mg^{2+} -bound or Mg^{2+} -free enzyme is used. However, there remains the possibility that a different residue in the same peptide has been labelled. Such a possibility has been ruled out by sequence studies (Fowler *et al.*, 1978).

Implications for the catalytic mechanism

The molecular nature of the methionine side chain precludes any acid-catalytic role for it. Nonetheless, it is alkylated by an enzyme-bound cation in which the electrophilic centre is at the precise site that, in a substrate, would be occupied by an atom to which electrophilic catalysis was being applied. Kinetic studies (Sinnott & Souchart, 1973) and studies of intermediates at low temperature (Fink, 1977) have indicated that, during the hydrolysis of aryl galactosides by Mg^{2+} -enzyme, a change in the conformation of the ES complex takes place. We have suggested (Sinnott & Withers, 1974; Withers, 1977) that this conformation change is associated with the correct placing of the acid-catalytic group. That this group is not captured by a carbonium ion centre at the precise place of its action is in accord with this hypothesis. A likely candidate for this acid-catalytic group is Tyr-501. Pro-499 might interrupt a sequence of, say, β -sheet and thus act as a 'hinge' for the conformation change.

We thank Dr. D. E. Games, University College, Cardiff, for running the field-desorption mass spectra on the Cardiff instrument. We are also indebted to Professor M. C. Whiting for advice on the preparation, properties and handling of alkylaryltriazenes and to Dr. A. V. Fowler for the merodiploid strain of *E. coli*. Financial support from the Science Research Council and I.C.I. Plant Protection is gratefully acknowledged, as is an equipment grant-in-aid from The Royal Society.

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