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Preliminary communication

Affinity labelling of β-D-galactosidase from *Escherichia coli* with *o*-nitrophenyl β-D-[6-^{3}H]galactopyranoside*

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The enzyme-catalyzed hydration[2] of D-galactal and the enzyme-catalyzed hydrolysis of β-D-galactopyranosides are similar as far as the products are concerned. In the case of hydration of D-galactal, a covalent, 2-deoxy-D-*lyxo*-hexosyl ("2-deoxy-D-galactosyl")-enzyme intermediate could be isolated[1]. As shown by kinetic studies[3] and initial-burst experiments[4], hydrolysis of *o*NPGal*** also proceeds through a (not necessarily covalent) D-galactosyl-enzyme that seems to be present in appreciable proportion. These similarities led us to assume the same type of intermediate in both reactions, and to try affinity labelling by denaturation using *o*NPGal, which is one of the best-investigated substrates[5] for β-D-galactosidase. Affinity labelling by quenching had previously been conducted with sucrose phosphorylase from *Pseudomonas saccharophila*[6,7], levansucrase (EC 2.4.1.10) from *Bacillus subtilis*[8], and β-D-glucosidase (EC 3.2.1.21) from *Aspergillus wentii*[9].

D-[6-^{3}H]Galactose was prepared from 1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose[10] (37 μmol) by reduction with NaB^3H_4 (9.26 μmol, 54 Ci/mmol) in absolute ethanol (1 mL), and subsequent hydrolysis[11]. After dilution with D-galactose, *o*NP[6-^{3}H]Gal was synthesized according to the method described for the unlabelled product[12]. Incubation of β-D-galactosidase from *Escherichia coli* with *o*NP[6-^{3}H]Gal at 0° under conditions of initial substrate-saturation was interrupted, immediately after mixing the components, by denaturation with guanidium chloride. The denaturated enzyme could be separated from material of low molecular weight by precipitation, and repeated washing with water.

The product so obtained was submitted to SDS-disc electrophoresis. Radioactivity was associated only with the protein (see Fig. 1), proving the covalent attachment of the ligand. Labelling of the enzyme above background could be achieved only so long as *o*NP[6-^{3}H]Gal was not yet completely hydrolyzed (see Fig. 2). Although hydrolysis of the substrate at the concentrations of enzyme used is extremely rapid, even at 0°, time-dependent incorporation of radioactivity could be measured, and this allowed extra-

*Several errors in a preceding paper[1] were due to publication of uncorrected proofs.
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***Abbreviations used: *o*NPGal, *o*-nitrophenyl β-D-galactopyranoside; EDTA, (ethylenedinitrilo)-tetraacetic acid; and SDS, sodium dodecyl sulfate.

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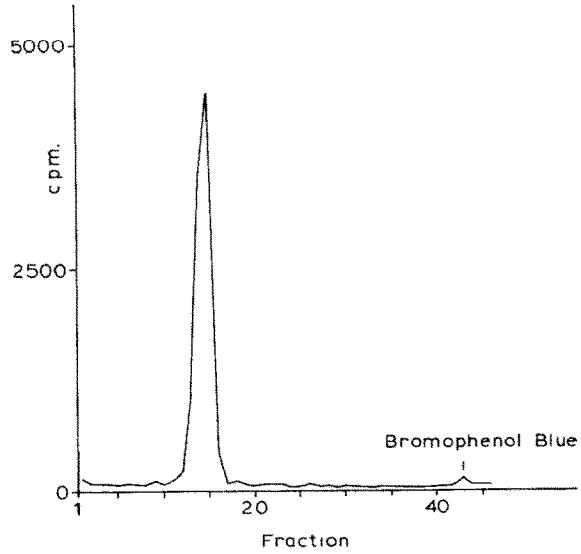
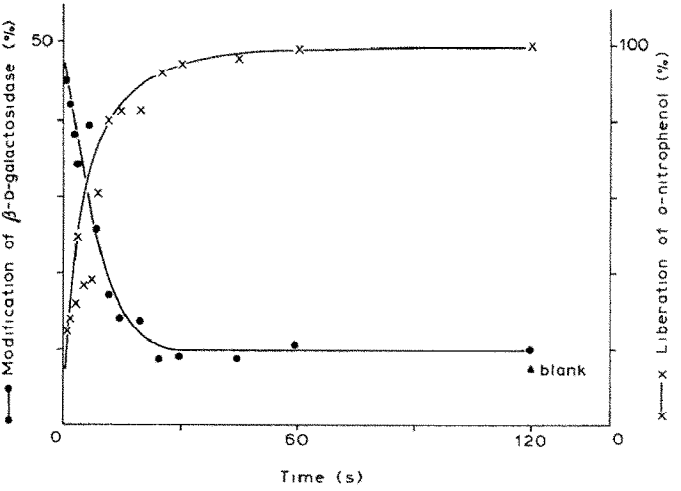


Fig. 1. Distribution of radioactivity after SDS–poly(acrylamide) gel electrophoresis of β-D-galactosidase labelled with *o*NP[6-^{3}H] Gal. [A solution (10 μL) of β-D-galactosidase (8 mg/mL) in 50mM sodium potassium phosphate buffer, pH 6.8, containing 1mM $MgCl_2$, was mixed at 0° with a solution (10 μL) of *o*NP[6-^{3}H] Gal (196 mCi/mmol; 1.58 mg/mL) in the same buffer. After an incubation time of <1 s, hydrolysis of *o*NP[6-^{3}H] Gal was quenched by addition of a solution (200 μL) of 8M guanidine hydrochloride and 10mM EDTA in 0.1M sodium phosphate buffer, pH 7.0. The protein was precipitated by dilution with ice-cold water (1 mL), and centrifuged. The supernatant liquor was discarded, and the protein was washed with ice-cold water (3 × 1 mL); no more radioactivity could be removed by further washing. The discontinuous, SDS–poly(acrylamide) gel electrophoresis[12] was performed in tubes (6 mm i. d. × 170 mm) according to O'Farrell[13]. The total acrylamide concentration of the gels was 7.5%, at a ratio of acrylamide:bis(acrylamide) of 97.3: 2.7. The length of the separation gels was 110 mm. Samples were applied after heating for 1 min at 95°. The temperature during electrophoresis was maintained at 10°; the current was kept constant at 1 mA/gel tube. After electrophoresis, one gel was cut into 2-mm-broad slices, in which the radioactivity was determined. A second gel was fixed with 12.5% trichloroacetic acid, and stained with Coomassie Brilliant Blue R 250.]



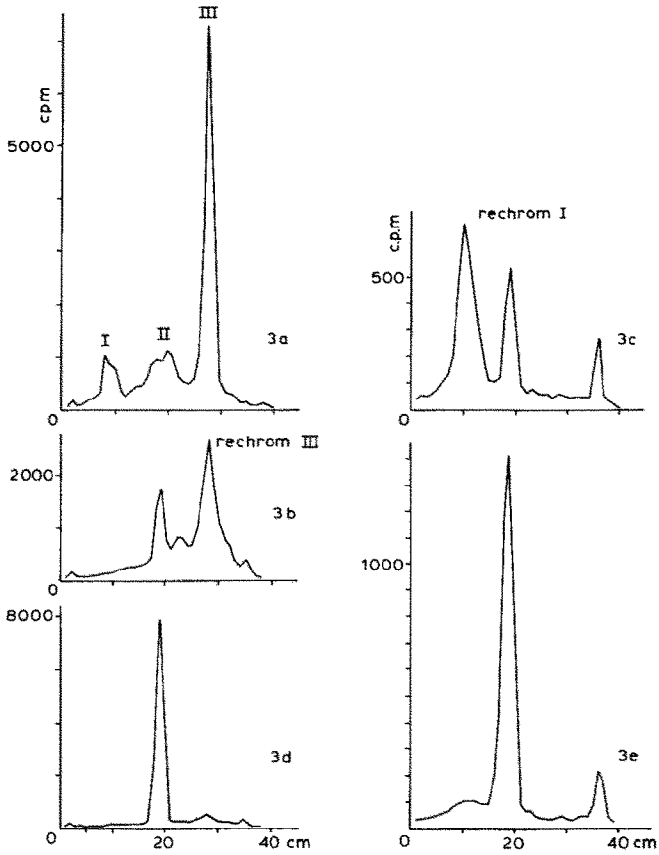


Fig. 3. Paper chromatography of products of papain digestion. [Labelled β-D-galactosidase was prepared as described in the legend to Fig. 1. A solution of papain (0.5 mg/mL) in 0.1M sodium citrate buffer, pH 6, containing 1mM EDTA and 1 mM 1,4-dithioerythritol, was added, to give a protein concentration of 5 mg/mL. The suspension was shaken for 24 h at 30°, and the solution applied to a sheet of Whatman No. 1 paper, which was then dried. Chromatography was performed in 1:1:1 (v/v) pyridine–butanol–water, adjusted to pH 5.8 with acetic acid. A strip (1 cm wide) of the chromatogram was cut into pieces (1 cm^2), and the attached radioactivity was measured by liquid scintillation counting. The radioactive products I and III (see Fig. 3a) so localized were eluted from the rest of the chromatogram, and the eluates freeze-dried. The samples were dissolved in 50mM sodium potassium phosphate buffer, pH 6.5, containing 1mM $MgCl_2$. Other parts of the samples were adjusted to pH 10, or treated with β-D-galactosidase (1 U), and kept for 1 h at 30°. These, as well as untreated samples, were rechromatographed, and analyzed as already described. Fig. 3b, untreated sample III; treatment at pH 10 did not change the pattern (chromatogram not shown). Fig. 3c, untreated sample I; treatment with β-D-galactosidase did not change the pattern (chromatogram not shown). Fig. 3d, sample III after treatment with β-D-galactosidase. Fig. 3e, sample I after treatment at pH 10.]

Fig. 2. Kinetics of modification of β-D-galactosidase by *o*NP[6-^{3}H]Gal. [Key: •—•, 14 samples (10 μL each) of a solution of β-D-galactosidase (11.1 mg/mL) were incubated with *o*NP[6-^{3}H]Gal as described in the legend to Fig. 1, except that denaturation of the enzyme was performed after incubation times of <1 s up to 100 s. The radioactivity bound to the protein was determined by liquid scintillation counting. x–x, Hydrolysis of *o*NP[6-^{3}H]Gal was measured spectrophotometrically at 405 nm after addition of saturated disodium hydrogenphosphate solution (100 μL) to the supernatant liquor. Hydrolysis is expressed as per cent of the final value.]

polation to zero time, when ~45% of the subunits of the enzyme had been modified. In contrast to the covalent modification of β-D-galactosidase by D-[6-^{3}H]galactal, the onset of labelling is much too fast for detection by any means available to us.

The remaining background radioactivity, corresponding to ~10% modification of subunits, was also found when the enzyme was incubated with D-[6-^{3}H]galactose or D-[U-^{14}C]glucose, or if the enzyme was denatured before incubation with *o*NP[6-^{3}H]-Gal. We assume that this is caused by nonspecific adsorption of radioactive material to the protein. The labelled protein was degraded for 24 h by papain without further treatment. Three radioactive components were separated by paper chromatography. One of these showed the same mobility as D-galactose (see Fig. 3a). The other radioactive components were not stable, and lost D-[6-^{3}H]galactose, as shown by rechromatography (see Fig. 3b, 3c). This process could be markedly accelerated by treatment with alkali for the slowest-moving (see Fig. 3e), or by treatment with β-D-galactosidase for the fastest-moving, component (see Fig. 3d). Both showed no increased lability against α-D-galactosidase from green coffee-beans.

These results suggest the modification of β-D-galactosidase by *o*NP[6-^{3}H]Gal at two different amino acid side-chains, which could occur when an intermediately formed, enzyme-bound, D-galactosyl cation reacts, during denaturation, with at least two, nearby, competing groups of the enzyme. A less likely explanation would be a partial trans-D-galactosylation of a covalently attached, D-galactosyl unit during denaturation of the native, D-galactosyl-enzyme intermediate.

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