LABELLING OF THE ACTIVE SITE OF β-GALACTOSIDASE BY *N*-BROMOACETYL β-D-GALACTOPYRANOSYLAMINE

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1. Introduction

N-Bromoacetyl β-D-galactopyranosylamine was designed as an affinity label for *lac*-permease [1] which has a reactive sulphydryl group at its substrate binding site [2]. The observed irreversible inactivation of the active transport of *lac*-permease substrates by this reagent is, however, not site-specific since active transport mediated by another permease, α MG-permease, is also inactivated [3]. In the course of our investigations of *lac*-permease, this reagent was found to inactivate β-galactosidase in intact *E. coli* cells [4].

We describe here the inactivation of purified β-galactosidase by *N*-bromoacetyl β-D-galactopyranosylamine. One mole of reagent is bound to the enzyme per mole of site inactivated. The described labelling procedure should facilitate the study of the peptide sequence in the vicinity of the active site of this enzyme.

2. Materials and methods

2.1. *Reagents*

N-Bromoacetyl β-D-galactopyranosylamine and *N*-bromoacetyl L-fucopyranosylamine were prepared as described [1]. Tritium-labelled *N*-bromoacetyl β-D-galactopyranosylamine was prepared with 2-^{3}H-bromoacetic acid purchased from The Radiochemical Centre, Amersham. The product melted at 180° with decomposition and was radiochemically pure when analyzed by paper chromatography with butanol–ethanol–water (4:1:1, by volume).

2.2. *The enzyme*

β-Galactosidase was purified from *E. coli* K12 strain 3300 essentially as described [5, 6] and was stored at 4° under 40% saturated ammonium sulphate solution of pH 6.0 containing 5% sodium chloride, 0.002 M magnesium chloride and 0.05 M sodium phosphate buffer.

The enzyme was analyzed in a tris-acetate buffer, pH 7.5, of the following composition: 0.01 M tris-acetate, 0.1 M sodium chloride, 0.01 M 2-mercapto-ethanol and 0.01 M magnesium chloride. The ratio of its absorbance at 280 to 260 nm was 1.97. The enzyme sedimented as a single component in a Spinco model E analytical ultracentrifuge with a sedimentation coefficient of 14.9 (protein concentration, 7.3 mg/ml; temperature, 22°). The enzyme was homogeneous in disc gel electrophoresis (7.5% at pH 8.3 maintained at 4°, 3 mA per column, staining with Coomassie Bleu). Enzyme activity was 480 μmoles/min/mg.

2.3. *Enzyme assay*

Hydrolysis of *o*-nitrophenyl β-galactopyranoside (ONPG) forms the basis of the assay. This was carried out in a solution of pH 7.0 containing 0.1 M sodium phosphate, 0.001 M $MgCl_2$, 0.1 M 2-mercaptoethanol and 0.70 g ONPG per liter of solution. Release of *o*-nitrophenol was followed in a Cary recording spectrophotometer at 420 nm using a 1 cm light path. The temperature of the assay was 28 ± 0.5°.

Enzyme activity is expressed in μmoles of *o*-nitrophenol produced in one minute by 1 mg protein in the conditions of the assay. Protein concentration was calculated from its absorbance, $A^{1\%}_{1\,cm}$ 280 nm = 20.9 [5]. For calculation of *o*-nitrophenol

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concentration, the molar absorbance, $\epsilon_{420\,nm}$, of 5200 was used for the *o*-nitrophenolate ion and the concentration *o*-nitrophenol at pH 7.0 was calculated by assuming a pK_a of 7.2.

2.4. *Enzyme inactivation and labelling*

Inactivation of enzyme was carried out at 33° in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.001 M magnesium chloride. In following the time course of inactivation 10 μl samples were transferred from the reaction mixture containing the alkylating reagent directly to the assay mixture and activity was determined as described. In a labelling experiment, after addition of the radioactive galactosyl reagent, samples were removed at predetermined times and immediately dialysed against cold buffer, pH 7.5, of the following composition: 0.01 M tris-acetate, 0.1 M sodium chloride, 0.01 M 2-mercaptoethanol and 0.01 M magnesium chloride. Dialysis was continued for up to 24 hr with several changes of buffer. The samples were then analyzed for enzymic activity, and for radioactivity. Counting rates were measured in a Packard liquid scintillation spectrometer.

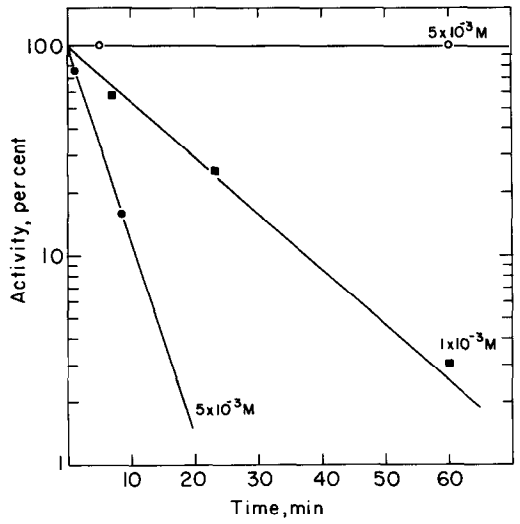

Fig. 1. Inactivation of β-galactosidase by *N*-bromoacetyl glycosylamine compounds. The full signs are for the galactosyl reagent, the open circles for the fucosyl reagent. Specified molar concentrations are of reagent in each experiment.

Table 1
Recovery of label in inactivation experiments.

Experiment 1		Experiment 2	
Sites inactivated %	moles label/mole inactivated site	Sites inactivated %	moles label/mole inactivated site
16	2.5	18	1.1
36	2.4	48	0.7
75	2.0	78	0.7

Two inactivation procedures were tested. In experiment 1 galactosyl reagent was used; in experimental 2, treatment with the galactosyl reagent was preceded by treatment with the fucosyl reagent. Samples were removed at predetermined times and excess reagent was removed by dialysis as described in Methods. Inactivation was calculated from the observed activity and the initial activity of the enzyme which was 480 μmoles/min/mg. An equivalent weight of 135,000 per site was used in the calculations.

3. Results and discussion

Inactivation of enzyme by *N*-bromoacetyl β-D-galactopyranosylamine followed first order kinetics and led to complete inactivation of enzyme (fig. 1). *N*-Bromoacetyl L-fucopyranosylamine did not inactivate the enzyme under the same conditions (fig. 1).

When enzyme was inactivated by treatment with 0.001 M radioactive galactosyl reagent, the label was slightly in excess of 2 equivalents of reagent per site inactivated (table 1, experiment 1). However, when the enzyme was first incubated with 0.005 M cold fucosyl reagent for 10 min and then with 0.001 M radioactive galactosyl reagent, a stoichiometry of 1:1, between sites inactivated and label introduced, was approached (table 1, experiment 2).

With this labelling procedure, it should be possible to isolate labelled peptides from the vicinity of the active site of the enzyme. This is an especially attractive possibility, since there are many mutants of β-galactosidase in which kinetic properties differ from those of the parent strain [7]. Work along these lines is now in progress in our laboratory.

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Additional note

While this manuscript was reviewed we found that the point of attachment of the site-specific label is a methionine sulphur. Details of experimental procedure will be published shortly.

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