LABELLING OF *LAC*-PERMEASE

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1. Introduction

N-Bromoacetyl β-D-galactopyranosylamine has been shown to inactivate *lac*-permease mediated accumulation of lactose in *E. coli* [1] *. The mechanism of inactivation of lactose uptake by this reagent was, however, not established. It was shown subsequently that *N*-bromoacetyl galactosylamine inactivates β-galactosidase by alkylating a methionyl residue near the active site of the enzyme [2]. Recently it was shown that it is possible to obtain β-galactosidase in a form which is not sensitive to the above reagent by growing a methionineless strain of *E. coli* in the presence of norleucine [3]. Using thus modified *E. coli* cells we have found that *N*-bromoacetyl galactosylamine inactivates *lac*-permease mediated flux of *o*-nitrophenyl β-D-galactoside in cells still able to hydrolyze the galactoside after treatment with the reagent [4]. This is evidence that in whole cells the reagent reacts with the *lac*-permease protein.

In this communication we report findings which demonstrate that *N*-bromoacetyl β-D-galactosylamine reacts with the *lac*-permease component of *E. coli* membranes. The number of lactose-binding sites in the membrane is 9600 per cell.

2. Materials and methods

2.1. *Membrane preparation*

A *lac*-permease constitutive strain of *E. coli*, ML308, was grown under aeration to a density of approx. 5×10^8 cells/ml in a 50 l fermentor on a salts medium "56" with 0.4% glycerol [5]. A quantity of 20 g of packed cells was washed with 0.01 M Tris buffer, pH 7.4, in a refrigerated Servall centrifuge. Cells were then suspended in 2 l of cold 20% sucrose solution in 0.33 M Tris buffer, pH 8.0. 100 ml of 0.1 M EDTA (pH 8.0) was added gradually to the suspension of bacteria gently agitated in an ice-water bath and this was followed by addition of 10 ml of lysozyme solution (5 mg/ml, 10 000 units/mg, Worthington Biochemical Corp., Freehold, N.J.). Fragility of the spheroplasts was determined by diluting the suspension 1:10 with distilled water and measuring the turbidity in a spectrophotometer. After the turbidity reached a constant value (approx. 5 min), the suspension was sedimented in a GSA rotor of a Servall refrigerated centrifuge for 10 min at 8000 rpm. The sedimented cells were suspended in 1.2 l of cold distilled water which contained $MgCl_2$ (0.002 M) and 5 mg of DNAase I (2500 units/mg, Worthington Biochemical Corp., Freehold, N.J.). The suspension was then sedimented in the same rotor for 5 min at 4000 rpm to remove unbroken cells. Membranes were collected by sedimentation of the supernatant for 15 min at 8500 rpm, as above. The membrane preparation was washed in 0.1 M sodium phosphate buffer, pH 7.0, and finally suspended in the same buffer. Membrane concentration was 17.3 mg dry matter/ml.

2.2. *Labelling procedure*

One ml of membrane suspension in 0.1 M sodium phosphate buffer, pH 7.0, in a heavy-walled 12 ml Servall centrifuge tube was incubated with tritium-labelled *N*-bromoacetyl β-D-galactopyranosylamine prepared as described [2]. Reaction was terminated by addition of 2 μl of 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.) followed by dilution of

* In this reference active compounds were in fact 2 and 4 and not 3 and 4 as printed.

Table 1
Labelling of an *E. coli* membrane preparation with *N*-bromoacetyl β-D-galactosylamine.

Time of contact (min)	Label incorporated			Inhibition of label incorporation by lactose (%)
	No addition (cpm)	Lactose present (cpm)	Inhibited by lactose (cpm)	
10	12900	4900	8000	62
30	18900	10200	8700	46
60	22500	14200	8300	37

Membranes (17.3 mg/ml) were incubated with 5×10^{-3} M reagent at room temperature (26°). Where lactose was present it was added to the membrane suspension, to a concentration of 10^{-1} M, 5 min before addition of the reagent. Reactions were terminated as described in Methods.

the suspension with 10 ml of the cold phosphate buffer. Membranes were sedimented for 15 min at 10 000 rpm in a SS-34 rotor of a Servall refrigerated centrifuge. Sedimented membranes were washed twice with 10 ml portions of cold buffer. They were then suspended in 5 ml of cold water to which 5 ml of 10% trichloroacetic acid was added with stirring. This was sedimented as above for 10 min at 7000 rpm. Sediment was washed once with 10 ml of cold 5% trichloroacetic acid, then with an ethanol–ether mixture (3:1) and finally with absolute ether. The air dried precipitate was dissolved in 0.2 ml of Soluene (Packard Instrument Comp., Downers Grove, III.) and was taken up and transferred quantitatively into counting vials with 20 ml of toluene based scintillating fluid. Counting rates were measured in a Packard liquid scintillation spectrometer and percent quenching was determined by the channel ratio method. Specific activity of tritium-labelled reagent in presence of 0.2 ml Soluene was 22 000 cpm/nmole; counting efficiency was 45% without membranes and 40% with membranes.

Table 2
Specificity of the lactose-binding sites in membrane of *E. coli*.

Disaccharide (0.1 M)	Inhibition of label incorporation, as per cent of the inhibition by 0.1 M lactose
Melibiose	100
Maltose	0
Cellobiose	50

The labelling procedure in the absence and in the presence of a disaccharide was as described in table 1. Time of contact with the reagent was 20 min.

3. Results and discussion

The extent of alkylation of *E. coli* membranes by *N*-bromoacetyl β-D-galactosylamine is small even on prolonged exposure (table 1). At 5×10^{-3} M reagent concentration the labelling of the lactose binding sites is complete in 10 min. At such short times and with a relatively high reagent concentration the lactose-binding sites account for 62% of the incorporated label. At lower reagent concentration the reaction should be even more specific.

The identity of the lactose-binding sites in the membrane of *E. coli* with *lac*-permease is demonstrated in table 2, where inhibition of galactosyl reagent incorporation by a number of disaccharides is compared. Melibiose, which is also a substrate of *lac*-permease [5], inhibits the incorporation of the label to the same degree as lactose. Maltose does not inhibit it at all and cellobiose inhibits it partially.

From results in table 1 the number of *lac*-permease sites in *E. coli* membrane can readily be calculated.

The quantity of label which was inhibited by lactose from incorporation into the membrane corresponds to 4.3×10^{-10} moles/17.3 mg of dry membrane material. If the dry weight of membrane is 20% of the dry weight of an *E. coli* cell and if the weight of a cell is 3.25×10^{-10} mg, this gives 9600 lactose-binding sites per cell. A similar number of *lac*-permease sites was found by labelling with *N*-ethyl maleimide of sites protected by such *lac*-permease substrates as thio-digalactoside or melibiose [6]. Another method of estimating *lac*-permease in the isolated membrane is by direct binding of thio-digalactoside [7]. In both these methods however, lactose offers no protection [8,7].

References

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