2,6-ANHYDRO-1-DIAZO-1-DEOXY-D-*GLYCERO*-L-*MANNO*-HEPTITOL: A SPECIFIC BLOCKING AGENT FOR THE ACTIVE SITE OF β-GALACTOSIDASE*

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Received 10 November 1975

1. Introduction

It is generally agreed upon that the enzymic cleavage of a β-galactosidase is triggered by protonation of the glycosidic oxygen [1]. This step is performed by a functional group 'AH' in the protein which is acidic enough to donate a proton to an acetal oxygen and close enough to do so in the catalytic process. Finding this functional group is of main importance for understanding the enzyme's action. Several attempts have been made so far to prove the identity of 'AH' by labelling the active site [2,3,4].

In this note the synthesis of 2,6-anhydro-1-deoxy-1-diazo-D-*glycero*-L-*manno*-heptitol (**1**) is described. (**1**) was conceived as an ideal compound for specifically labelling the protonating group 'AH'. It is shown to react smoothly and specifically with β-galactosidase from *E. coli*.

2. Materials and methods

2.1. *Reagents*

2,6-Anhydro-1-deoxy-1-diazo-D-*glycero*-L-*manno*-heptitol (**1**) was prepared in solution by treating 3,4,5,7-tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-nitrosoacetamido-D-*glycero*-L-*manno*-heptitol (10 mg= 23 μM) in methanol (50 μl) with N $NaOCH_3$ solution (10 μl). The solution shows infrared absorption at 4.8 μm which is characteristic for diazo compounds. The solution is stable for several minutes.

3,4,5,7-Tetra-*O*-acetyl-2,6-anhydro-1-deoxy-1-nitroso-acetamido-D-*glycero*-L-*manno*-heptitol was synthesized as follows: To a solution of 1-acetamido-3,4,5,7-tetra-*O*-acetyl-2,6-anhydro-1-deoxy-D-*glycero*-L-*manno*-heptitol [5] (15.0 g) in CCl_4 (150 ml) and glacial acetic acid (150 ml) anhydrous sodium acetate was added with stirring. Nitrogen dioxide (approx. 9 g) was bubbled through the suspension until the green colour persisted for at least 15 min. At this point $CHCl_3$ (400 ml) was added and the reaction mixture was stirred into icecold, saturated aqueous bicarbonate solution (2 liters). As soon as the neutralisation was complete the organic layer was separated, washed with water (2 X 200 ml) and dried over $MgSO_4$. Evaporation in vacuo yielded a solid residue which was recrystallised from ether/light petroleum.

Yield: 14.0 g (86%), melting point: 85–87°C,

$[\alpha]_{578}^{25}$ −43.9° (c=1, $CHCl_3$). $C_{17}H_{24}O_{11}N_2$ (432.39)

Calculated: C 47.22 H 5.59 N 6.48

Found: C 47.23 H 5.48 N 6.59

β-Galactosidase from *E. coli* and β-glucosidase from sweet almond emulsin were purchased from Boehringer, Mannheim, isopropyl-D-thio-galactoside (IPTG) from Serva, Heidelberg.

2.2. *Incubation of β-galactosidase and β-glucosidase with* 1

The buffer used for the incubation experiments with **1** was tris-HCl (0.2 M, pH 8.0) containing additional 10^{-2} M 2-mercaptoethanol per liter in the case of β-galactosidase only. To the enzyme solution (0.2 ml containing 50 μg protein) a freshly prepared solution of **1** (10 μl) was added by rapid injection

*Part I of the series: Uncommon results of glycosidase action.

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using a Hamilton syringe. Evolution of nitrogen was observed immediately*. Between two injections nitrogen bubbles were removed by centrifugation. Experiments were performed at 30°C.

2.3. *Enzyme assay*

Enzymic hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside or *o*-nitrophenyl-β-D-glucopyranoside was performed at 30°C in a pH 6.8 solution containing 0.1 M sodiumphosphate and 10 mM substrate per liter. In the case of β-galactosidase the buffer contained additional 10 mM 2-mercaptoethanol and 1 mM $MgCl_2$ per liter. The assay was started by addition of the enzyme solution (0.05 ml of 0.2 M Tris buffer pH 8.0 in a total volume of 2.5 ml). The absorption of nitrophenol liberated was measured at 405 nm. Enzyme solutions containing the reversible inhibitor IPTG were dialysed prior to the assay.

*It could be shown that the main products of spontaneous decomposition in buffer are 2,6-anhydro-D-*glycero*-L-*manno*-heptitol and 1-deoxy-D-*galacto*-heptulose [6]. The products as well as the solvent methanol have no effect on β-galactosidase activity in the concentrations used.

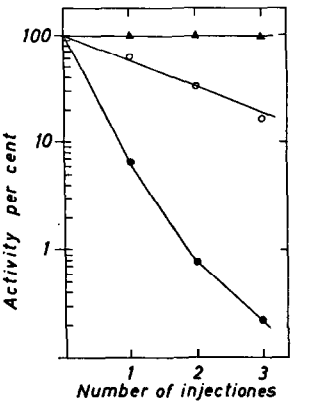

Fig.1. The full circles show inactivation of β-galactosidase by repeated injections of 1 in methanolic solution. The open circles show the same experiment when the reversible inhibitor IPTG (60 mM) is present. The triangles show no effect of 1 on β-glucosidase activity.

2.4. *Dialysis*

All dialyses were carried out at 4°C against 0.1 M sodiumphosphate buffer pH 6.8 containing 10 mM 2-mercaptoethanol and 1 mM $MgCl_2$ per liter.

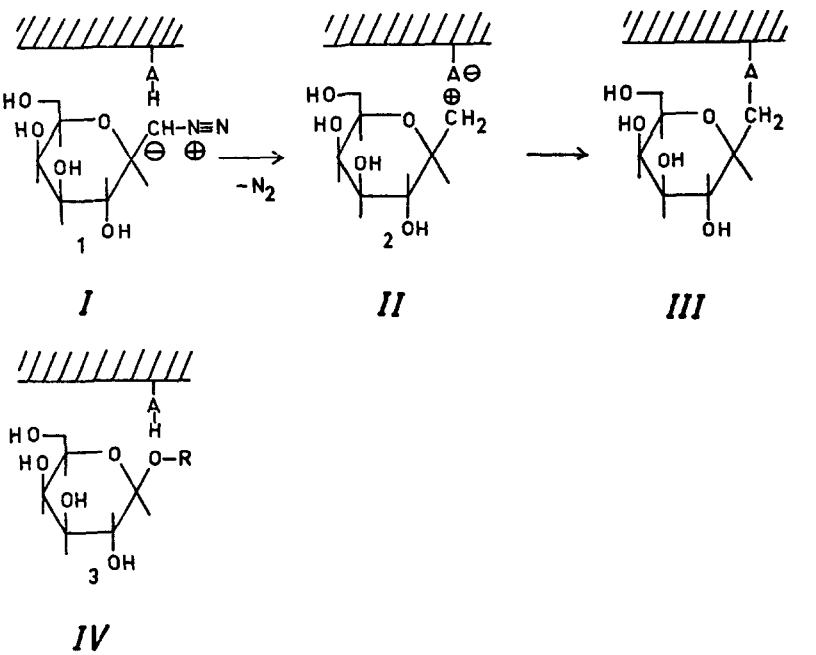

Fig.2. Interaction in the enzyme-substrate complex *I* with substrate 1 produces a highly reactive carbenium ion 2 in complex *II*. This may yield the covalent complex *III*. For comparison enzyme–substrate complex *IV* with an ordinary substrate 3 is depicted underneath.

3. Results

Since **1** in methanolic solution starts decomposing rapidly when added to water or aqueous buffer it is understandable that inactivation of β-galactosidase is not complete even when a large excess of inactivator is used. Portionwise treatment was found to be more effective and therefore comparably small quantities of **1** were sufficient for nearly total inactivation of β-galactosidase (fig.1). The inactivation could not be reversed by dialysis. Analogous treatment of β-glucosidase with the inactivator had no effect on the enzyme activity (fig.1). In order to show that **1** actually blocks the binding site for the substrate, inactivation of β-galactosidase was performed in the presence of the reversible inhibitor IPTG. IPTG clearly protects the enzyme by competing with the irreversible inhibitor **1** for the binding site (fig.1).

4. Discussion

The results suggest that the glyconic part of **1** is held by the enzyme in much the same way as the glyconic part of a β-galactoside (**3**). This must bring the strongly nucleophile carbon-1 of **1** equally close to the protonating group 'AH' (*I*) as the glycosidic oxygen of a natural substrate (*IV*).

Protonation of carbon-1 in complex *I* creates a highly reactive carbenium ion **2** in complex *II* after spontaneous elimination of nitrogen. **2** reacts with a nucleophile nearby. It is reasonable to assume that this should be the anion of 'AH' which thus causes its own labelling (*III*). Work is in progress to confirm this assumption.

Acknowledgement

We thank the Deutsche Forschungsgemeinschaft for financial support.

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