Biochem. J. (1971) **125**, 717–719
Printed in Great Britain

β-Galactosidase-Catalysed Hydrolysis of β-D-Galactopyranosyl Azide

By MICHAEL L. SINNOTT
Department of Organic Chemistry, University of Bristol, Bristol BS8 1TS, U.K.

(*Received* 14 *May* 1971)

1. β-D-Galactopyranosyl azide is hydrolysed by the β-galactosidase of *Escherichia coli* to galactose and azide ion at a mechanistically significant rate. 2. Methyl 1-thio-β-D-galactopyranoside is a competitive inhibitor of the hydrolysis of the azide and of *o*-nitrophenyl β-D-galactopyranoside with K_i 1.8mM. 3. β-Galactosidase can thus hydrolyse a range of substrates of general structure β-D-galactopyranosyl-X(Y), where the atom X has a lone pair of electrons on which the enzyme may act as a Lewis or Brønsted acid, but in which the length of the bond cleaved varies significantly, which is inconsistent with the orbital steering hypothesis.

Barnett and his co-workers (Barnett, Jarvis & Munday, 1967*a*,*b*; Barnett & Jarvis, 1967) have reported that glycosyl fluorides are excellent substrates for glycoside hydrolases. The qualitative similarity of glycosyl azides and fluorides (Micheel & Klemer, 1961) made it seem probable that the azides would also be substrates, albeit worse ones because of their greater stability to both acids and bases. Investigations in progress made β-galactosidase a convenient enzyme.

METHODS AND MATERIALS

β-D-Galactopyranosyl azide (*I*). Reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (9.0g) in CaH_2-dried acetonitrile (9.0ml) with NaN_3 (6.0g) for 1.5h under reflux, followed by filtration, evaporation and twofold recrystallization from di-isopropyl ether, afforded 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl azide (2.0g; 25% yield), m.p. 95–96°C [Micheel & Klemer (1961) quote m.p. 96°C], ν (N_3) 2125cm^{-1} and ν (C=O) 1745cm^{-1} (CCl_4). Deacylation of the tetra-acetate (1.0g) with 0.01M-sodium methoxide (5.0ml) in methanol followed by evaporation of the solvent and recrystallization of the residue from ethanol yielded β-D-galactopyranosyl azide (0.33g; 73%), m.p. 150–152°C [Micheel & Klemer (1961) give m.p. 152°C], ν (N_3) 2140cm^{-1} and ν (OH) 3450 and 3300–3150cm^{-1} (Nujol).

o-Nitrophenyl β-D-galactopyranoside. This was recrystallized twice from water, m.p. 200–203°C (decomp.). Conchie & Levvy (1963) quote m.p. 193–194°C.

Methyl 1-thio-β-D-galactopyranoside. This was prepared from 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (Horton, 1963); it had m.p. 182–183°C (decomp.) after two recrystallizations from ethanol [Horton (1963) gives m.p. 174–175°C].

Enzymes. β-D-Galactopyranose dehydrogenase (lot no. 6329211), β-galactosidase from *Escherichia coli* (lot no. 7500408) and NAD^+ were purchased from Boehringer Corp. (London) Ltd. (London W.5, U.K.).

All enzyme-catalysed hydrolyses were performed at 25.0°C in 0.1M-sodium phosphate buffer, pH7.0, containing 1mM-$MgCl_2$.

Determination of azide ion. The u.v. absorption of N_3^- in 0.1M-sodium phosphate buffer, pH7.0, containing 1mM-$MgCl_2$ was utilized; at 245nm, the absorption minimum of proteins and covalent azides, ε (NaN_3) is 188 but ε (galactosyl azide) is 18. This differential absorption was also used to follow the kinetics of hydrolysis of β-D-galactopyranosyl azide (I). The yield of N_3^- on complete hydrolysis was measured for a solution (5.0ml) containing the azide (I) (14.6mg) after reaction with β-galactosidase (0.010mg) for 20h.

Determination of D-galactose. This was carried out by measuring the absorption at 340nm due to NADH (ε 6.22×10^{-3}) formed on oxidation with β-D-galactopyranose dehydrogenase. Use of a sample of the solution used for measurement of N_3^- in this assay resulted in an apparently non-quantitative yield (83%) of D-galactose, but this might have been an artifact of destruction of NADH at a rate comparable with mutarotation of the other forms of the sugar to the β-pyranose form. Consequently hydrolysis and oxidation were carried out in one operation: β-D-galactopyranose is the product of β-galactosidase-catalysed hydrolyses. Absorption of three solutions containing NAD^+ (1.0mg), β-D-galactopyranosyl azide (0.1057mg), β-D-galactopyranose dehydrogenase (0.005mg) and β-galactosidase (0.005mg) in the phosphate buffer described (3.0ml) in 1cm cells was monitored for 2h in a Unicam SP.800 spectrophotometer with a cell block thermostatically controlled at 25.0°C. NADH formation was not detectable during this time if the β-galactosidase was omitted.

Kinetic measurements. Kinetic parameters for the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside were determined in 1cm cells in the thermostatically controlled (water) (to ±0.3°C) cell compartment of a Hilger

and Watts Uvispek H700 single-beam spectrophotometer producing analogue extinction output through a Gilford recording attachment and a Sunvic 10S recorder. The hydrolysis was followed at 400nm, where ϵ (*o*-nitrophenol) is 2.33×10^3 in the medium used. K_m and $k_{cat.}$ values were determined from a linear least-squares treatment of plots of v against $v/[S]$. Quoted errors are standard errors on 13 points.

Other kinetic measurements were made in a Unicam SP.1800 spectrophotometer with a cell block thermostatically controlled (water) to ±0.1°C with a Tecam Tempunit circulating pump. Analogue extinction output was produced through a Servoscribe recorder. K_m and $k_{cat.}$ values for the azide were calculated as for the glycoside (eight points), but for the inhibition experiments rates were fitted to the expression for competitive inhibition:

$$v = \frac{V_{max.}}{1+\frac{K_m}{[S]}\left(1+\frac{[I]}{K_i}\right)}$$

by using the FORTRAN IV program Spiral (Jones, 1970), which iteratively minimizes square residuals in v directly. Concentrations of methyl 1-thio-β-D-galactopyranoside varied from 0 to 30mM, those of *o*-nitrophenyl β-D-galactopyranoside from 0.021 to 0.83mM, and those of β-D-galactopyranosyl azide from 0.67 to 10mM. For each substrate–inhibitor combination rates were measured at each of five inhibitor concentrations for each of five substrate concentrations.

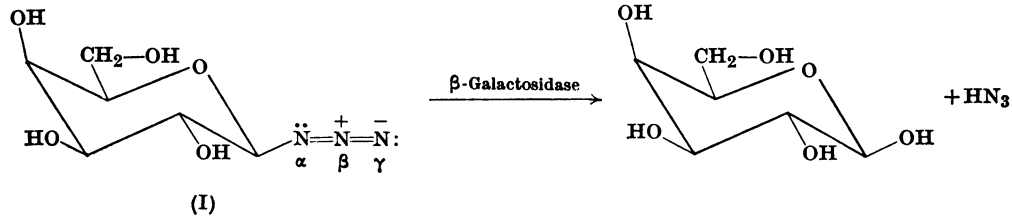
Active-site molarities were calculated assuming pure protein, $E^{0.1\%}_{1cm}$ 2.1 at 280nm and a molecular weight per active site of 1.35×10^{-5} (Viratelle, Tenu, Garnier & Yon, 1969). The activity of the protein for the accurate determination of Michaelis parameters for *o*-nitrophenyl galactoside was 670000 units/mg and for the inhibition experiments and the azide hydrolysis 470000 units/mg. One unit (Cohn, 1957) is defined as that quantity of enzyme which will hydrolyse 1nmol of *o*-nitrophenyl β-D-galactopyranoside in 1min at 28°C in 0.1M-sodium phosphate buffer, pH7.0, containing 0.1M-2-mercaptoethanol, when the concentration of substrate is 0.70g/l.

RESULTS AND DISCUSSION

The enzyme-catalysed hydrolysis of the azide (I) yielded the expected products, D-galactose (97%) and N_3^- (103%) (Scheme 1). The galactosyl azide is stable indefinitely in the buffer used.

The enzyme-catalysed hydrolysis exhibited Michaelis–Menten kinetics with K_m 2.83±0.28mM and $k_{cat.}$ 26s^{-1} ($[E]_0$ 247nM; activity 470000units/mg); *o*-nitrophenyl β-D-galactopyranoside, the best substrate known, was hydrolysed with K_m 0.100±0.007mM and $k_{cat.}$ 847s^{-1} ($[E]_0$ 2.47nM; activity 670000units/mg); the values for this substrate are in excellent agreement with those of Tenu, Viratelle, Garnier & Yon (1971).

The relatively high $k_{cat.}$ value for the azide [comparable with that for phenyl β-D-galactopyranoside (Tenu *et al.* 1971)] and the moderately low K_m value [comparable with that for lactose (Wallenfels & Malhotra, 1961)] imply that the hydrolysis of this compound occurs through the operation of the same functional groups at the same active site as that of conventional substrates. This was confirmed by competitive inhibition experiments: phenyl 1-thio-β-D-galactopyranoside and *o*-nitrophenyl 1-thio-β-D-galactopyranoside are known to be competitive inhibitors of *E. coli* β-galactosidase (Wallenfels & Malhotra, 1961), but are unsuitable for use with the azide as they absorb strongly at 245nm: their methyl analogue was therefore used, with some assurance that the inhibition would also be competitive. For the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside K_i (methyl 1-thio-β-D-galactopyranoside) is 1.81±0.06mM, K_m is 0.112±0.03mM and $V_{max.}$ is 719±10nM·s^{-1} ($[E]_0$ 1.24nM; activity 470000units/mg). For the hydrolysis of β-D-galactopyranosyl azide, K_i (methyl 1-thio-β-D-galactopyranoside) is 1.79±0.08mM, K_m is 2.56±0.14mM and $V_{max.}$ is 6900±170nM·s^{-1} ($[E]_0$ 247nM; activity 470000units/mg). Quoted errors are standard deviations. The excellent fit of the results to the expression for competitive inhibition (95% confidence regions extend maximally over 5 standard deviations; values in the parameter correlation matrix do not exceed 0.88; there are no discernible trends in the signs of the residuals), and in particular the low errors in $V_{max.}$ confirm that the inhibition is competitive. The identity of the K_i values for methyl-1-thio-β-D-galactopyranoside determined against the two substrates establishes that the azide is hydrolysed at the same site as *o*-nitrophenyl β-D-galactopyranoside.



(I)

Scheme 1.

In addition to alkyl and aryl β-D-galactopyranosides and β-D-galactopyranosyl azide, β-galactosidase will also hydrolyse β-D-galactopyranosyl fluoride (von Hofsten, 1961) and 1-thiol-β-D-galactopyranosides of strongly acidic thiophenols such as thiopicric acid (Wallenfels, Schadel, Schillinger & Hengstenberg, 1964). The enzyme can thus catalyse the heterolytic cleavage of C–O, C–F, C–N and C–S bonds, all of which will have different bond lengths. Bond lengths in model compounds are (Sutton, 1958, 1965): $H_2C(-OCH_3)_2$, 1.42 Å; H_3C–F, 1.385 Å; H_2FC–F, 1.36 Å; H_3C–N_3, 1.47 Å; H_3C–SH, 1.82 Å; $(p BrC_6H_4-)_2S$, 1.75 Å. This toleration by the enzyme of appreciable changes in an important molecular dimension of the substrate seems difficult to reconcile with recent theories (Storm & Koshland, 1970) that the catalytic efficiency of enzymes may be related to a very precise definition of angles of approach of nucleophilic and electrophilic catalytic groups (cf. Capon, 1971).

Little is known about the mechanism of action of glycosidases; only for lysozyme are the catalytic groups known with certainty, and for this enzyme the widely repeated mechanism (e.g. Chipman & Sharon, 1969) is based on model building and the postulation of distortion and electrostatic effects that have been shown (Bruice & Dunn, 1970; Guidici & Bruice, 1970) to be of negligible importance in model systems. It does, however, seem probable that some form of electrophilic or acidic 'pull' on the aglycone is necessary for its removal (Capon, 1969), and in accord with this hypothesis all the leaving groups so far discovered (^-OR, F^-, N_3^-, ^-SAr) have a lone pair of electrons, to which this 'pulling' species can co-ordinate, on the atom directly attached to the pyranose ring. The N_3^- ion has lone pairs on both the α- and the γ-nitrogen atoms, so in the enzymic hydrolysis of galactosyl azides the 'pull' must occur at the α-nitrogen atom, the analogue of the exocyclic oxygen atom of oxygen glycosides.

I thank the Science Research Council for support, Dr H. Gutfreund and Professor M. C. Whiting for advice, and Dr P. J. England for enlightenment on the FORTRAN IV program Spiral.

REFERENCES

Barnett, J. E. G. & Jarvis, W. T. S. (1967). *Biochem. J.* **105**, 9P.

Barnett, J. E. G., Jarvis, W. T. S. & Munday, K. A. (1967*a*). *Biochem. J.* **103**, 699.

Barnett, J. E. G., Jarvis, W. T. S. & Munday, K. A. (1967*b*). *Biochem. J.* **105**, 669.

Bruice, T. C. & Dunn, B. M. (1970). *J. Am. chem. Soc.* **92**, 2410.

Capon, B. (1969). *Chem. Rev.* **69**, 407.

Capon, B. (1971). *J. chem. Soc.* B, p. 1209.

Chipman, D. M. & Sharon, N. (1969). *Science, N.Y.*, **165**, 454.

Cohn, M. (1957). *Bact. Rev.* **21**, 140.

Conchie, J. & Levvy, G. A. (1963). In *Methods in Carbohydrate Chemistry*, vol. 2, p. 334. Ed. by Whistler, R. L. & Wolfrom, M. L. New York: Academic Press Inc.

Guidici, T. A. & Bruice, T. C. (1970). *Chem. Commun.* p. 690.

Horton, D. (1963). In *Methods in Carbohydrate Chemistry*, vol. 2, p. 371. Ed. by Whistler, R. L. & Wolfrom, M. L. New York: Academic Press Inc.

Jones, A. (1970). *Comput. J.* **13**, 301.

Micheel, F. & Klemer, A. (1961). *Adv. Carbohyd. Chem.* **16**, 85.

Storm, D. R. & Koshland, D. E. (1970). *Proc. natn. Acad. Sci. U.S.A.* **66**, 445.

Sutton, L. E. (1958). *Spec. Publs chem. Soc.* no. 11.

Sutton, L. E. (1965). *Spec. Publs. chem. Soc.* no. 18.

Tenu, J. P., Viratelle, O. M., Garnier, J. & Yon, T. (1971). *Eur. J. Biochem.* **20**, 363.

Viratelle, O. M., Tenu, J. P., Garnier, J. & Yon, T. (1969). *Biochem. biophys. Res. Commun.* **37**, 1036.

von Hofsten, B. (1961). *Biochim. biophys. Acta*, **48**, 159.

Wallenfels, K. & Malhotra, O. P. (1961). *Adv. Carbohyd. Chem.* **16**, 239.

Wallenfels, K., Schadel, P., Schillinger, E. & Hengstenberg, W. (1964). *Angew. Chem.* (*int. Edn*), **3**, 450.