# THE β-GALACTOSIDASE-CATALYZED HYDROLYSIS OF o-NITROPHENOL-β-D-GALACTOSIDE AT SUBZERO TEMPERATURES: EVIDENCE FOR A GALACTOSYL-ENZYME INTERMEDIATE

Anthony L. Fink and Kim J. Angelides

Division of Natural Sciences, University of California, Santa Cruz, California 95064.

Received April 2,1975

Summary The reaction of β-galactosidase (E. coli K12) with o-nitrophenyl-β-D-galactoside has been investigated over the temperature range +25° to -30° using 50% aqueous dimethyl sulfoxide as solvent. At temperatures below -10° turnover becomes very slow and a burst of o-nitrophenol is observed. Such a burst indicates the existence of a galactosyl-enzyme intermediate whose breakdown is rate-limiting and provides a means of determining the active site normality. The Arrhenius plot for turnover is linear in the -25 to +25° range with $E_a$ = 26 ± 3 kcal/mole. The presence of the 50% DMSO had no effect on $K_m$ but caused a small decrease in $k_{cat}$.

β-Galactosidase from E. coli K12 is a tetramer of MW 520,000 which has been the subject of several recent investigations concerning its mechanism of action, and a recent review (1-3). From $H_2{}^{18}O$ and other studies it is clear that the bond that is cleaved in the enzyme-catalyzed hydrolysis of β-galactosides is the one between the anomeric carbon (C1) and the glycosyl oxygen (4). Relatively little information is available concerning the residues involved in the catalytic reaction. A methionine residue (5) and possibly a histidine (6) have been implicated. The formation of a galactosyl-enzyme intermediate has been suggested based on the retention of configuration in the products, and transglycosidation in the presence of alcohols (1), as well as product partitioning ratios in the presence of alcohol (3). When the hydrolysis of o-nitrophenyl-β-D-galactoside

(ONPG) was studied in the presence of methanol the rate of the enzyme-catalyzed reaction initially increased with increasing methanol concentration and then levelled off (7). This was interpreted to indicate a change in rate determining step from solvolysis of the galactosyl-enzyme at low methanol concentration to its formation at high methanol concentrations (7). However, alternative explanations are possible (8).

From analogy with lysozyme (9), and the non-enzymatic hydrolysis of glycosides it has been suggested that β-galactosidase-catalyzed hydrolyses may involve a carbonium ion intermediate (10). An alternative mechanism involving a covalent galactosyl-enzyme intermediate and a double displacement reaction, with or without participation by the C2-OH, has also been proposed (1, 4). Recently a scheme involving a conformational change, intimate ion-pair, and a covalent intermediate has been suggested (2).

We have been interested in applying our technique of using very low temperatures to slow enzyme-catalyzed reactions down sufficiently to allow the accumulation of transient intermediates (11-13) to β-galactosidase for two main reasons. First, virtually no information is available concerning the effects of the aqueous organic solvent systems necessary to maintain fluid solutions in the desired temperature range (to -100°C) on oligomeric enzymes. Secondly, relatively little mechanistic information is available for this enzyme which has considerable potential in such studies because of the knowledge concerning its genetic aspects and the potential for obtaining mutants. Further, it is likely that β-galactosidase could serve as a prototype for the many other glycoside hydrolases.

This communication reports some of our preliminary findings,

in particular direct evidence for a galactosyl-enzyme intermediate which may be readily accumulated and trapped at temperatures below -20° in 50% aqueous dimethyl sulfoxide.

Material and Methods. β-galactosidase was isolated and purified from E. coli K12 by standard procedures (14) and stored at 0°. o-Nitrophenyl-β-D-galactoside (ONPG) was obtained from Sigma. Aqueous dimethyl sulfoxide (DMSO) solutions were made by mixing cold acetate buffer (0.05 M) of the appropriate pH with the desired volume of DMSO at 0° and adding KCl to give $\mu$ = 0.1 M, and pH values in the 6-7 range. Solutions of 50% and 65% (v/v) were used in these studies and made up on a weight basis. The dimethyl sulfoxide was purified by vacuum distillation at 37° after drying with calcium hydride. The 0% DMSO runs were done in tris buffer (0.05 M), pH 7.0 and $\mu$ = 0.1 M (KCl). Buffers were made either 1 or 50 mM in $MgCl_2$. Kinetic and o-nitrophenol burst measurements were made using a Cary 118C spectrophotometer. Low temperature kinetic experiments were performed as described previously (12). Standard kinetic assay conditions for β-galactosidase activity were pH 7.0 (tris), 25°, and [S] = $2 \times 10^{-2}$ M. $V_{max}$ and $K_m$ were determined using the initial velocity method. The low-temperature burst experiments were carried out in the following manner. Solutions of ONPG ($2 \times 10^{-2}$M) in 50% DMSO, pH 6.1 were cooled to the desired temperature and scanned from 550 to 415 nm. Temperature was monitored and maintained within ± 0.15° during the entire experiment. An aliquot of enzyme (stock solution diluted 3-fold with 50% DMSO buffer) was added and carefully mixed at time = 0. Repeated wavelength scans were then taken for several minutes. Control runs were also done in which either enzyme or substrate was omitted. No spectral changes were observed in these control experiments. The data were analyzed by plotting

$A_{440}$ vs. time. The slope of the resulting line is proportional to $V_{max}$, and the intercept at time = 0 yields the magnitude of the burst of ONP. In the determination of $k_{cat}$ values for $E_o$ were obtained from burst experiments at -22°. All experiments were run at least in duplicate.

Results. Initial experiments were aimed at determining the effect of DMSO on the β-galactosidase-catalyzed hydrolysis of ONPG. At pH 7.0, in 65% aqueous DMSO considerably reduced catalytic activity was observed at both 25° and 0°, apparently due to decreased stability of the enzyme. At 50% aqueous DMSO the enzyme was stable at 0°, losing activity at the linear rate of 7% per day. Comparison of the values of $K_m$ and $k_{cat}$ in 0% and 50% aqueous DMSO for typical conditions are shown in Table I. Figure 1 shows the Arrhenius plot obtained from the hydrolyis of ONPG in 50% aqueous DMSO, pH 6.1 which gives an energy of activation of 26 ± 3 kcal/mole. Under the experimental conditions the turnover

Table I. Catalytic parameters for the hydrolysis of ONPG, pH 7.1, 25° in 0 and 50% aqueous DMSO, 50 mM $MgCl_2$.

| Solvent (% DMSO) | $K_m$ ($M \times 10^4$) | $k_{cat}$ $sec^{-1}$ |
|---|---|---|
| 0 | 4.6 ± 0.5 | $1.6 \times 10^3$ |
| 50 | 5.0 ± 0.5 | $3.4 \times 10^2$ ($4.8 \times 10^2$) [a] |

a. At pH 6.1

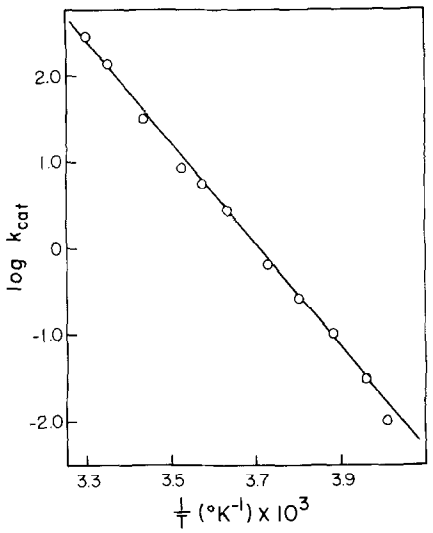


Figure 1. Arrhenius plot for the β-galactosidase-catalyzed hydrolysis of ONPG, pH 6.1, 50% DMSO, $S_o = 2 \times 10^{-2}$ M, $E_o = 3.7 \times 10^{-7}$ M, $k_{cat}$ in $sec^{-1}$, 50 mM $MgCl_2$.

---

reaction becomes very slow at temperatures below -20° even at very high enzyme concentration. At such temperatures rapid bursts of o-nitrophenol were observed. Both the magnitude of the bursts (Fig. 2) and the initial rates under substrate saturating conditions were directly proportional to the enzyme concentration.

Discussion. The utility of using subzero temperatures to allow the detection of an enzyme-substrate intermediate is readily apparent in this work. The presence of bursts of o-nitrophenol, stoichiometric with enzyme concentrations, under conditions where turnover is negligible indicates the presence of a galactosyl-enzyme intermediate and is in accord with a minimum scheme of the form

$$E + S \rightleftarrows ES \xrightarrow{k_2} \begin{matrix} EG \\ + \\ P_1 \end{matrix} \xrightarrow{k_3} E + P_2 \qquad \text{(Scheme 1)}$$

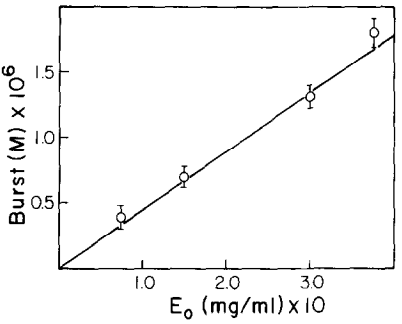


Figure 2. Magnitude of o-nitrophenol bursts as a function of enzyme concentration. pH 6.1, 50% DMSO, -22°, $S_o$ = 2 x $10^{-2}$ M, 50 mM $MgCl_2$. Under these experimental conditions the burst reaction was over within a few seconds and no attempts were made to measure its rate.

---

where ES = Michaelis Complex, EG = galactosyl-enzyme, $P_1$ = o-nitrophenol and $P_2$ = galactose. The presence of such bursts when turnover is essentially zero means that $k_2 >> k_3$. Consequently the temperature dependence of $k_{cat}$ in Fig. 1 corresponds to that of $k_3$. Apparently $k_2$ has a much lower energy of activation and is consequently much less affected by temperature changes. Based on studies of transglycosylation in the presence of alcohols at 25°, it has been reported that $k_2$ is not substantially greater than $k_3$ (7, 15). This report is the first direct evidence for the accumulation of a galactosyl-enzyme intermediate. Subsequent studies will be directed at elucidating the nature of the intermediate.

Since the bursts of o-nitrophenol should be stoichiometric with the amount of active enzyme, under conditions where turnover is negligible, they may be used as the basis for active site normality titrations (16). The results in Fig. 2 indicate that the enzyme preparation was 61% active. The values of $K_m$ obtained in this study (Table I) are in accord with reported

values of $K_m$ for ONPG which vary considerably, due no doubt to the different experimental conditions and the sensitivity of the reaction to various metal ions (1). The presence of 50% DMSO has no effect on $K_m$ (Table I) for the reaction. A 50% decrease in $k_{cat}$ would be expected if there were no specific binding site for water in the hydrolysis of the galactosyl-enzyme. The five-fold decrease in $k_{cat}$ at pH 7.1 in 50% DMSO compared with 0% DMSO (Table I) appears to be due to changes in the pH optimum and effect of $Mg^{+2}$ in the presence of 50% DMSO. For example at 1 mM $Mg^{+2}$ only the anticipated two-fold decrease based on the reduced water concentration was observed. Detailed studies of the effect of DMSO on the structural and catalytic properties of this enzyme will be reported shortly. The value for $k_{cat}$ obtained in the absence of DMSO (Table I) is higher than than previously reported (7) and presumably reflects the fact that crystalline preparations of β-galactosidase appear to be only 60% active based on the active site normality (Fig. 2).

Acknowledgement. This investigation was supported by a grant from the National Science Foundation.

References

1. Wallenfels, K. & Weil, R. (1972) Enzymes 7, 617
2. Sinnott, M. L. & Souchard, I.J.L. (1973) Biochem. J. 133, 89.
3. Stokes, T. M. & Wilson, I. B. (1972) Biochemistry 11, 1061.
4. Wallenfels, K. & Malhotra, O. P. (1961) Adv. Carbo. Chem. 16, 239
5. Yariv, J., Wilson, K. J., Hildesheim, J. & Blumberg, S. (1971) FEBS Letts. 15, 24.
6. Proctor, M. H. (1962), Biochem. Biophys. Acta. 59, 713.
7. Tenu, J. P., Viratelle, O. M., Garnier, J., & Yon, J. (1971) European J. Biochem. 20, 363.
8. Sinnott, M. L. & Viratelle, O. M. (1973) Biochem. J. 133 81.
9. Imoto, T., Johnson, L.N., North, A. C. T., Philips, D.C. & Rupley, J. A. (1972), Enzymes 7, 665.
10. Lee, Y. C. (1969), Biochem. Biophys. Res. Comm. 35, 161.
11. Fink, A. L. & Good, N. E. (1974) ibid., 58, 126.

12. Fink, A. L. (1973), Biochemistry 12, 1736.
13. Fink, A. L. (1975), submitted for publication.
14. Craven, G. P., Steers, E. & Anfinsen, C. B. (1965), J. Biol. Chem. 240, 2668.
15. Van Der Groen, G., Wouters-Leysen, J., Yde, M. & De Bruyne, C. K. D. (1973), European J. Biochem. 38, 122.
16. Bender, M. L. Begue-Canton,M. L., Blakely, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kezdy, F. J., Killheffer, Jr., J. V., Marshall, T. H., Miller, C. G., Roeske, R. W. and Stoops, J. K. (1966), J. Amer. Chem. Soc. 88 , 5890.