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Galactosyl Transfer Catalyzed by Thermostable β-Glycosidases from *Sulfolobus solfataricus* and *Pyrococcus furiosus*: Kinetic Studies of the Reactions of Galactosylated Enzyme Intermediates with a Range of Nucleophiles[1]

Inge Petzelbauer, Barbara Splechtna, and Bernd Nidetzky[2]

Division of Biochemical Engineering, Institute of Food Technology, University of Agricultural Sciences (BOKU), Muthgasse 18, A-1190 Vienna, Austria

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The transfer of a galactosyl group from an enzyme to a number of neutral primary alcohols, phenol and azide has been studied during the reactions at 80°C of thermostable β-glycosidases from *Sulfolobus solfataricus* (SsβGly) and *Pyrococcus furiosus* (CelB) with 2-nitrophenyl β-D-galactopyranoside or lactose (4-*O*-β-D-galactopyranosyl D-glucopyranose) as substrates. The rate constant ratios, k_{Nu}/k_{water}, for partitioning of the galactosylated enzyme intermediates between reaction with nucleophiles (k_{Nu}, M^{-1} s^{-1}) and water (k_{water}, s^{-1}) have been determined from the difference in the initial velocities of the formation of 2-nitrophenol or D-glucose, and D-galactose. The results show that hydrophobic bonding interactions contribute ≈8 kJ mol^{-1} to the stabilization of the transition state for the reaction of galactosylated enzyme intermediates of SsβGly and CelB with 1-butanol, compared to the transition state for the enzymatic reaction with methanol. The leaving group/nucleophile binding sites of SsβGly and CelB appear about 0.8 times as hydrophobic as *n*-octanol. Values of k_{Nu}/k_{water} for reactions of galactosylated SsβGly with ethanol and substituted derivatives of ethanol show no clear dependence on the pK_a of the primary hydroxy group of these nucleophiles in the pK_a range 12.4–16.0. The binding of phenol with the galactosylated enzyme intermediates of SsβGly and CelB occurs in a form that is mainly nonproductive pertaining to β-galactoside synthesis. Neither enzyme catalyzes galactosyl transfer to azide ion. A model is proposed for the interaction of neutral nucleophiles at an extended acceptor site of the galactosylated en-zymes.

Key words: binding, nucleophile, kinetic partitioning, reactivity, transgalactosylation.

Hyperthermostable β-glycosidases from the Archaea *Sulfolobus solfataricus* (SsβGly) (*1*) and *Pyrococcus furiosus* (CelB) (*2*) catalyze the hydrolysis of a wide variety of β-glycosides of the general form β-Glx-OR, where Glx is the glycon moiety and RO(H) is the leaving group (*3–6*). β-glycoside hydrolysis catalyzed by both enzymes proceeds with retention of configuration at C-1 of Glx. β-retaining glycosyl transfer catalyzed by β-glycosidases is generally thought to occur through a two-step reaction mechanism that is promoted by a catalytic nucleophile and a general acid/base, and involves the formation of a covalent α-glycosyl enzyme intermediate (E-Glx) (reviewed in Refs. 7 and *8*). The catalytic nucleophiles of SsβGly (Glu387) and CelB (Glu372) are defined through the known structure/function relationships of family 1 glycosyl hydrolases (*9*), and were identified by site-directed mutagenesis (*6, 10*) and through mechanism-based inactivation (*11*).

Transglycosylation is a commonly observed, diagnostic feature of enzyme-catalyzed glycosyl transfer with retention and occurs because E-Glx partitions between reaction with water and reaction with other nucleophiles present in bulk solution (Scheme 1) (*7, 8, 12*). The transfer of Glx to acceptors other than water leads to the formation of new β-glycosides in a kinetically controlled reaction. While complete hydrolysis of the substrate usually predominates in dilute solutions of reactants, a significant change in the partitioning of E-Glx towards transglycosylation can be achieved by decreasing the water activity through the addition of an organic co-solvent or using very high concentrations of acceptors. The good stabilities of SsβGly and CelB in non-natural media and the possibility of working at high temperatures have been exploited in biotechnology to improve β-glycoside yield and productivity in transglycosylation reactions catalyzed by archaeal "thermozymes" (*13–15*). However, unless mutant glycosyl hydrolases with drastically impaired capabilities of glycosidic bond cleavage are utilized in the synthesis reaction (*8, 15, 16*), the newly formed β-glycosides are hydrolyzed with time, and those that are degraded slowly eventually accumulate at the end. Therefore, it is generally difficult to deduce the specificity of E-Glx for reaction with nucleophiles by simply determining

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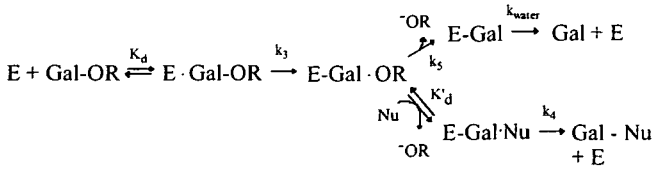
[2] To whom correspondence should be addressed. Phone: +43-1-36006-6274, Fax: +43-1-36006-6251, E-mail: nide@edv2.boku.ac.at

Abbreviations: SsβGly, β-glycosidase from *Sulfolobus solfataricus;* CelB, β-glycosidase from *Pyrococcus furiosus;* oNPGal, 2-nitro-phenyl-β-D-galactoside; oNP, 2-nitro-phenol.



Scheme 1. **Nucleophilic competition in the proposed three-step reaction of thermostable β-glycosidases from *S. solfataricus* and *P. furiosus* with 2-nitro-phenyl-β-D-galactoside (oNPGal), where E is the enzyme, Gal-OR is oNPGal, -OR is 2-nitro-phenol/phenolate and Nu is a nucleophile.**

the product ratios at extended reaction times. Clearly, measurements of initial velocities of hydrolysis and glycosyl transfer under defined reaction conditions are required to obtain a better understanding of the specificity for glycosyl transfer catalyzed by SsβGly and CelB.

The experimentally observable specificities of glycosylated SsβGly and CelB for reactions with acceptors of the general form R-OH, where R is a non-substituted or substituted straight-chain alkyl group or aromatic ring, will be the product of several factors, among them most importantly the intrinsic reactivity of R-OH towards E-Glx, which is commonly related to the pK_a value of the OH group (*7, 17*), and the stabilization of the transition state of glycosyl transfer by noncovalent bonding of E-Glx to R (*7, 8, 18–21*). It is, therefore, of considerable interest to quantitate the contributions to transition state-stabilization energy, which are due to binding interactions, and distinguish them from those that can be described in structure/reactivity relationships. Since active-site metals are not present in SsβGly (*22*) and CelB (*23*), noncovalent interactions with R-OH will probably involve hydrophobic bonds and hydrogen bonds, and aromatic interactions. Binding interactions of D-glucose and galactosylated SsβGly and CelB have recently been shown to increase the nucleophilic reactivity of the sugar 45-fold compared to that of methanol (*24*). Likewise, the effective leaving group ability of D-glucose in lactose is increased 10^3-fold compared to that of methanol in methyl-β-D-galactopyranoside, in spite of the very similar pK_a values of both leaving groups (*24*). (The estimated pK_a value of the 4-OH of D-glucose is 14.8 while that of methanol is 15.1).

In continuation of our previous studies of transgalactosylation to sugar acceptors catalyzed by SsβGly and CelB (*24–26*), the present paper reports nucleophile trapping experiments studying the reactions of galactosylated enzyme intermediates with a range of neutral nucleophilic alcohols differing in the pK_a of the primary OH group, and their nonpolar bonding capability with the enzymes. Attempts were made to trap the galactosyl-enzyme intermediates with the very nucleophilic azide ion. The results clearly reveal the presence of an expanded nucleophile/leaving group binding site in both enzymes. Individual nucleophiles differ markedly in the extent to which nonproductive complex formation occurs upon their addition to the proposed binding site. The binding site provides hydrophobic and aromatic interactions with the nucleophile, which contribute ≥19 kJ/mol of stabilization energy that is specific to the transition state of galactosyl transfer. By contrast, there is no observable dependence on the alcohol pK_a of the partitioning of galactosyl-enzyme intermediates between reaction with alcohol and water. This finding may reflect the fact that electronic interactions in the transition of galactosyl transfer are clouded by noncovalent binding interactions on the one hand, and the contribution of general base catalysis to the addition of these alcohols to the reactive intermediate on the other. The results have implications for the synthesis of new β-D-glycosides by transglycosylation using hyperthermostable SsβGly and CelB.

EXPERIMENTAL PROCEDURES

Materials—2-Nitro-phenyl-β-D-galactopyranoside (oNPGal) was from Sigma, lactose was from Fluka. All other chemicals were of reagent purity and obtained from Sigma through local suppliers. Alkyl alcohols were >98% pure and used without further purification.

Enzymes—Recombinant β-glycosidases from *S. solfataricus* and *P. furiosus* were produced using the expression of plasmid-encoded structural genes in *Escherichia coli*, as described previously for both enzymes (*4, 27*). The enzymes were purified by a two-step procedure involving first, the thermoprecipitation of mesophilic bacterial protein at 80°C for 30 min, and second, anion exchange chromatography on a MonoQ column (Amersham-Pharmacia) using elution with a linear gradient of 0 and 1 M NaCl in 20 mM potassium phosphate buffer, pH 6.8. The purified SsβGly and CelB had specific hydrolase activities of ≈600 U/mg and ≈2,000 U/mg, respectively, determined using the enzyme assay with oNPGal as the substrate (see below).

Assays—The hydrolase activities of SsβGly and CelB were determined at 80°C and pH 6.5 using 15 mM oNPGal as the substrate with discontinuous measurement of the release of 2-nitro-phenol (oNP) at 405 nm. D-Glucose was determined by an enzymatic assay based on using glucose oxidase and peroxidase (*24*). D-Galactose was measured enzymatically using the NAD^+-dependent oxidation of D-galactose by galactose dehydrogenase (Boehringer Mannheim).

Kinetic Analysis—A three-step reaction mechanism of hydrolysis of oNPGal by SsβGly and (probably) CelB is assumed and shown in Scheme 1 (*24*). The first irreversible step of the mechanism, which is represented by the rate constant k_3, is cleavage of the glycosidic linkage. Dissociation of the leaving group from the complex with the galactosylated enzyme (k_5) and the addition of water to the enzyme-galactose intermediate (k_{water}) can be kinetically significant reaction steps. In the absence of exogenous nucleophiles, the expression for the turnover number (*28, 29*) becomes

$$k_{cat} = 1/(1/k_3 + 1/k_5 + 1/k_{water}) \quad (1)$$

while the catalytic efficiency is given by the relation

$$k_{cat}/K_m = k_3/K_d \quad (2)$$

where K_d is the rate constant ratio k_2/k_1.

In the presence of nucleophiles that bind to an acceptor site at the galactosylated enzyme with a dissociation constant K'_d, the expression for k_{cat} is

$$k_{cat} = 1/(1/k_3 + 1/k'_{water}) \quad (3)$$

where k'_{water} is the net rate constant for the total conductance through the top and bottom paths of the mechanism shown in Scheme 1 (*24, 28*). k'_{water} is given by the relation

$$k'_{water} = (k_5 + k'_{Nu})/(1 + k_5/k_{water} + k'_{Nu}/k_4) \quad (4)$$

where k'_{Nu} is another net rate constant described by the relation

$$k'_{Nu} = k_4\,[Nu]/K'_d \quad (5)$$

where [*Nu*] is the acceptor concentration and K'_d is the dissociation constant of the binary complex of the galactosylated enzyme and *Nu*.

Using Eqs. 4 and 5 one can rearrange Eq. 3 to yield

$$k_{cat} = k_3\,(k_5 + k_4\,[Nu]/K'_d)/\{k_3 + k_5 + k_3k_5/k_{water} + (k_3 + k_4)\,[Nu]/K'_d\} \quad (6)$$

Considering Eqs. 3 to 6, it is interesting to analyze theoretically the possible effects of added nucleophiles on the observed value of the turnover number. Added nucleophile will cause a decrease in k_{cat} when $k_4 < k_{water}$ or $k_4 < k_5$. When k_4 is greater than k_{water} and k_5, the effect of added nucleophiles on k_{cat} will mirror whether rate limitation is due to k_3 (no change), or k_{water} or k_5 (increase) (*24, 28*).

Galactosyl Transfer—Enzymatic reactions were carried out in 50 mM sodium phosphate buffer, pH 6.5, at 80 ± 2°C using oNPGal as the substrate in a total volume of 0.5 ml, and gentle mixing of the reactants with an Eppendorf Thermomixer model 5436 with instrument settings at 400 × rpm. The buffer containing the substrate was pre-incubated under these conditions for approx. 10 min, and the temperature was controlled externally before the reaction was started by the addition of enzyme (20 µl). The final enzyme concentration was ≤0.1 U/ml. A constant substrate concentration of 7.5 mM oNPGal was used. This substrate concentration reflects a useful compromise between the effort of maximizing the transfer of D-galactose to external alcohols but not the substrate (*24*) on the one hand, and the practical requirement for precisely measurable initial velocities of D-galactose release on the other. The formation of oNP and D-Gal was measured in samples (15 µl) taken over reaction times up to 1 h. The relationships of [oNP] and [D-Gal] against reaction time were linear up to 20 min unless substrate depletion was greater than 15%. Therefore, a standard reaction time of 5 min was used, and the rates of formation of oNP (v_{oNP}) and D-Gal (v_{Gal}) were calculated from measurements of the concentrations of oNP and D-Gal released. Values of v_{oNP} and v_{Gal} were determined in the absence and presence of acceptors (*17, 24, 26*), and the acceptor concentrations varied between 0.05 and 6 M, as indicated under "RESULTS." Transfer constants (k_{Nu}/k_{water}) were calculated from the slopes of straight lines in plots of the velocity ratio, v_{oNP}/v_{Gal}, against the acceptor concentration, according to the relationship in Eq. 7 derived from Scheme 1

$$v_{oNP}/v_{Gal} = 1 + k_{Nu}\,[Nu]/k_{water} \quad (7)$$

where v_{oNP} and v_{Gal} are the initial velocities based on the release of oNP and D-Gal, respectively. In Eq. 7, k_{Nu} equals k_4/K'_d and is a specificity constant for the reaction of E-Gal with Nu. The F-test at the 99% probability level confirmed the validity of the linear fit for the range of [*Nu*] shown in the respective figures. It allowed the elimination of data for v_{oNP}/v_{Gal}, generally observed at low [*Nu*], which apparently did not fit the straight line as required by the model given in Eq. 7. The goodness of fit for the lines shown in the figures is represented by the value of r^2, which was usually greater than 0.985. If not stated otherwise, the maximum concentration of the acceptor used for the determination of k_{Nu}/k_{water} inhibited v_{oNP} by less than 20% compared to a control that lacked the external acceptor. All measurements were carried out in duplicate and shown to be reproducible within an experimental error of ±10% for v_{oNP}/v_{Gal}. The reported results are the mean values of two independent determinations. When lactose (150–500 mM) replaced oNP-Gal as the substrate for the enzymatic reaction, experiments were carried out under otherwise identical conditions as described above, and the rates of release of D-Glc and D-Gal were measured.

RESULTS AND DISCUSSION

Reaction of Galactosylated Enzyme Intermediates and Hydrophobic Nucleophiles—During the reactions of SsβGly and CelB with oNPGal as the substrate, v_{oNP}/v_{Gal} increased in the presence of neutral primary alcohols of the form R-CH_2OH, where R is an unbranched alkyl chain, compared to the control reaction which did not contain the alcohol. The observed v_{oNP}/v_{Gal} was dependent on [R-CH_2OH], and the results for SsβGly and CelB are shown in panels (a) and (b) of Fig. 1, respectively. At low [R-CH_2OH], all v_{oNP}/v_{Gal} profiles showed an upward curvature, indicating the increment of the slope, $\Delta(v_{oNP}/v_{Gal})/\Delta$[R-$CH_2OH$], as [R-$CH_2OH$] was increased. With all alcohols studied, the slope approached a maximum value which then remained constant within the limits of experimental error for a certain concentration range of R-CH_2OH. The linearity for the range of [*Nu*] shown in Fig. 1 was confirmed by regression and statistical analyses (see the "EXPERIMENTAL PROCEDURES"). The value for the concentration of R-CH_2OH at which v_{oNP}/v_{Gal} began to show a linear dependence on [R-CH_2OH], or in other words, the maximum slope of the curve was reached, decreased with increasing size of the alkyl chain R, as summarized in Table I.

From Fig. 1 one can see that the ratio of v_{oNP}/v_{Gal} for the enzymatic reaction of SsβGly (panel a) and CelB (panel b) with oNPGal in the absence of alkyl alcohols was 2.6 and 1.8, respectively. The observed "initial" value of v_{oNP}/v_{Gal} was greater than 1 because of the efficient galactosyl transfer from the enzyme to the substrate oNPGal, which takes place with a partitioning ratio, k_{oNPGal}/k_{water}, of 125 and 97 M^{-1} for reactions catalyzed by SsβGly and CelB, respectively (*24*). The formation of transgalactosylation products originating from the reactions of galactosylated enzyme intermediates of SsβGly and CelB with oNPGal has been demonstrated recently using HPLC to analyze the product mixtures obtained during the course of the enzymatic conversion of oNPGal over time (*26*). A probable explanation for v_{oNP}/v_{Gal} profiles that are concave upward at low [R-CH_2OH], is the fact that oNPGal and R-CH_2OH compete for binding to the nucleophile binding site of the galactosyl-enzyme intermediate (E-Gal). We assume in Scheme 2 and discuss later that acceptor-mode binding of oNPGal occurs at an extended binding site of E-Gal, which provides interactions with both the D-galactose *and* the nitrophenol moiety of the substrate, now poised for reaction with E-Gal rather than glycosidic bond cleavage (*24*). Interactions of E-Gal and R-CH_2OH taking place at subsites +1 or +2 (using subsite nomenclature proposed in Ref. *30*, Scheme 2) will prevent the binding of oNPGal, but only that at subsite +1

will eventually be productive and lead to the transfer of D-galactose and thus an increase in v_{oNP}/v_{Gal}. If at low [R-CH_2OH], binding of the alcohol occurred preferentially at subsite +2 and at the same time inhibited the reaction of E-Gal with water, the shape of the curve in Fig. 1 would be explained.

Hydrophobicity of the Nucleophile Binding Site of Galactosylated SsβGly and CelB—The values of k_{Nu}/k_{water} for the reactions of galactosylated SsβGly and CelB with R-CH_2OH were determined from parts of the v_{oNP}/v_{Gal} profiles where v_{oNP}/v_{Gal} was linearly dependent on [R-CH_2OH]. Results are summarized in Table I. k_{Nu}/k_{water} increased with increasing size of the alkyl chain R and thus hydrophobicity of the alcohol. By using the relationship,

$$\Delta\Delta G^{\ddagger} = RT \ln [(k_{Nu}/k_{water})_R/(k_{Nu}/k_{water})_H]$$

the incremental Gibbs free energies of the transfer of R from the enzyme to water can be calculated, relative to H (methanol), where R is the gas constant (8.31441 J mol^{-1} K^{-1}) and T is the temperature (353.15 K). The data shown in Table I imply a significant contribution of hydrophobic interactions between R and E-Gal to a stabilization that is specific to the transition state of galactosyl transfer. The decrease in the activation energy for the reaction of galactosylated SsβGly and CelB was, respectively, 0.75 and 0.80 times smaller than the free energy of transfer of the alkyl group from water to *n*-octanol (ΔG_{trans} in Table I). ΔG_{trans} at 80°C was calculated using the expression, 2.303 RT π,

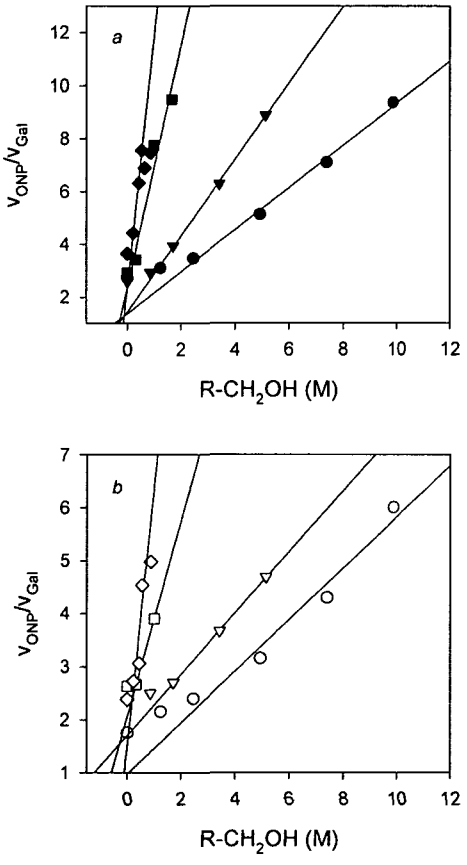
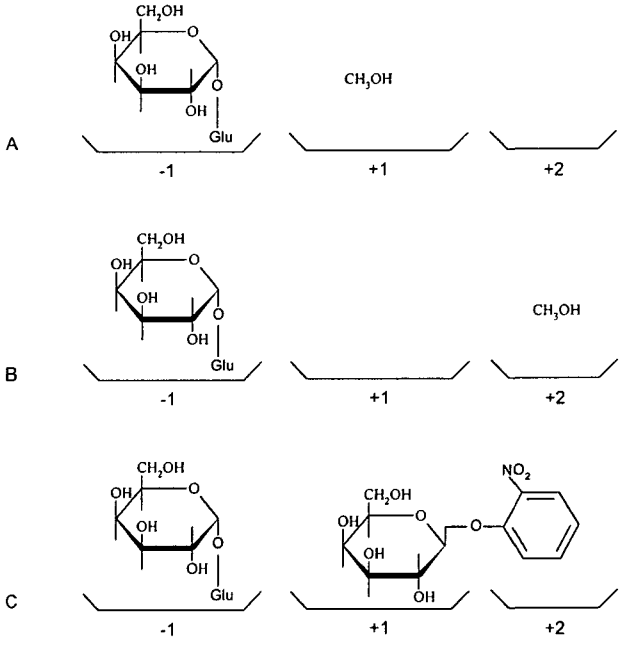


Fig. 1. **Transfer of D-galactose from the enzyme to hydrophobic, straight-chain primary alcohols determined from the partitioning of the galactosylated enzyme intermediates of SsβGly (a) and CelB (b) between reaction with the primary alcohol and water.** The constant concentration of oNPGal was 7.5 mM, and the reactions were carried out at 80°C and pH 6.5. Symbols denote methanol (circles), ethanol (triangles), propanol (squares), and butanol (diamonds).



Scheme 2. **Hypothetical organization into subsites of the active site of SsβGly or CelB, and occupancy of the subsites during enzymatic galactosyl transfer to acceptors bound at the proposed extended binding site for the leaving group/nucleophile.** The galactosyl residue at subsite –1 is covalently linked *via* an α-glycosidic bond to the carboxylate group of a glutamate residue of the protein: Glu387 in SsβGly (*10, 11*) and and Glu372 in CelB (*6*). Small nucleophiles, such as methanol, may bind productively at subsite +1 (A) or nonproductively at subsite +2 (B), whereas nucleophiles, such as oNPGal, bind at both subsites at the same time (C). Water may react with the galactosylated enzyme intermediate when a small nucleophile is bound nonproductively at subsite +2.

TABLE I. **Galactosyl transfer to neutral, primary alkyl alcohols catalyzed by SsβGly and CelB.** The reactions were carried out at 80°C and pH 6.5 using 7.5 mM oNPGal as the substrate.

Alcohol (R)	SsβGly				CelB		
	π[a]/ΔG_{trans} (kJ mol^{-1})[b]	Δ_{max} (M)[c]	k_{Nu}/k_{water} (M^{-1})	$\Delta\Delta G^{\ddagger}$ (kJ mol^{-1})[d]	Δ_{max} (M)[c]	k_{Nu}/k_{water} (M^{-1})	$\Delta\Delta G^{\ddagger}$ (kJ mol^{-1})[d]
Methanol (-H)	–	≈2	0.79	–	2.5	0.35	–
Ethanol (-CH_3)	0.5/3.4	≈1	1.44	1.8	1.8	0.52	1.2
Propanol (-CH_2-CH_3)	1.0/6.8	<0.2	4.54	5.1	<0.2	1.84	4.9
Butanol (-$(CH_2)_2$-CH_3)	1.5/10.1	<0.1	9.40	7.3	<0.1	4.93	7.8

[a]Hydrophobicity constant of R; [b]incremental Gibbs energy of transfer from *n*-octanol to water; [c]value for the acceptor concentration at which v_{oNP}/v_{Gal} is linearly dependent on [R-CH_2-OH]; [d]the calculation of $\Delta\Delta G^{\ddagger}$ is described in the text

where π is the hydrophobicity constant of the alkyl chain R (*31*). Plots of $\Delta\Delta G^{\ddagger}$ against ΔG_{trans} were reasonably linear for reactions catalyzed by SsβGly and CelB, with correlation coefficients of 0.965 and 0.988, respectively (not shown). This indicates that the nucleophile/leaving group binding sites of galactosylated CelB and SsβGly are 1.25 (=1/0.80) and 1.33 (=1/0.75) times less hydrophobic than *n*-octanol.

Effect of Hydrophobic Nucleophiles on the Rate of D-Galactoside Hydrolysis—The turnover number for the hydrolysis reaction of SsβGly with oNPGal is 1,300 s^{-1} (*24*), and was found to increase by a factor of between 1.9 and 2.2 in the presence of R-CH_2OH, independent of the chain length of R. However, the value of [R-CH_2OH] at which the observed k_{cat} was a maximum, decreased from >2 M to 0.3 M as R increased in size from methanol to butanol. Rate enhancement by added R-CH_2OH is indicative of a reaction mechanism of SsβGly in which the rate of degalactosylation (k_{water}) or the rate of the dissociation of the leaving group (k_5) partially limits the overall rate of hydrolysis of oNPGal, and R-CH_2OH reacts more rapidly with the galactosylated enzyme than does water, that is, k_4 is greater than k_{water} and k_5 (*7, 8, 24, 32–35*). Upon the addition of increasing concentrations of R-CH_2OH, the rate of reaction is expected to increase up to a point where it becomes limited by the rate of galactosylation (k_3), which in the absence of other effects of alcohols on protein structure, should be independent of the chain length of R. That was observed, suggesting a value for k_3 of ≈2,270–2860 s^{-1} (1.9–2.2 × k_{cat}).

D'Auria *et al.* (*36*) have recently interpreted time-resolved tryptophanyl fluorescence data of SsβGly recorded in the absence and presence of straight-chain primary alcohols such that the conformational flexibility of the protein was increased upon binding of the alcohols. They suggested that alterations in the structural dynamics of SsβGly induced by primary alcohols might explain their observation of an increase (by about 10%) in k_{cat} for the hydrolysis of oNPGal at 75°C when 80 mM 1-butanol was present (*36*). It is conceivable that the binding of 1-butanol stabilizes a more reactive conformation of the enzyme and thereby produces a rate-enhancing effect. In addition, if the enzymatic mechanism of SsβGly were expanded to accomodate a partly rate-limiting conformational change in enzyme structure (for which we have no evidence, however), a rate-enhancing effect of 1-butanol could arguably be ascribed to the increase in the rate of such a conformational change. To distinguish between a rate enhancement by added primary alcohols that is brought about by the specific acceleration of certain reaction steps of the kinetic mechanism in Scheme 1, and one that is due to global changes in protein strucure, we studied the effect of 2-propanol on the rate of hydrolysis of oNPGal catalyzed by SsβGly. Galactosyl transfer from E-Gal to 2-propanol takes place with a very small rate constant ratio, k_{Nu}/k_{water}, of <0.1 M^{-1} (*24*) and, therefore, the effect of 2-propanol on steps involved in degalactosylation (including k_5) should be minimal. The observed rate enhancement in the presence of 2-propanol was 1.6-fold, the maximum effect being seen at ≈2 M alcohol. This concentration was twice that needed to observe maximum 'activation' of SsβGly for the hydrolysis of oNPGal in the presence of 1-propanol. It is noteworthy that 1-propanol serves as a good acceptor of D-galactose from galactosylated SsβGly (*24*). We propose, therefore, that R-CH_2OH probably affects the SsβGly-catalyzed hydrolysis of oNPGal in two different ways, one of which is related to the kinetic scheme of the enzymatic reaction in the presence of external nucleophiles (Scheme 1), and the other accomodates the changes in protein structure brought about by the binding of hydrophobic alcohols (*36*, see below). With CelB, the observed rate enhancement of the hydrolysis of oNPGal by added R-CH_2OH, with R being -H, or -CH_3 to -$(CH_2)_2$-CH_3, was not greater than 1.3-fold and not significantly different from the observed rate enhancement for the same reaction in the presence of the incompetent nucleophile, 2-propanol.

Rate of Formation of D-Galactose in the Presence of Nucleophiles—From Scheme 1 we can derive the expression for the rate of formation of D-galactose in the presence of acceptors that intercept the galactosylated enzyme intermediate.

$$k_{cat}\,(\text{D-galactose}) = k_3k_5/\{k_3 + k_5 + k_3k_5/k_{water} + (k_3 + k_4)\,[Nu]/K'_d\} \quad (8)$$

Equation 8 predicts a decrease of k_{cat} (D-galactose) in the presence of external nucleophiles. Figure 2 shows the observed change in k_{cat} (D-galactose) as a function of [R-CH_2-OH], using three primary alcohols differing in R. The results clearly demonstrate that independent of the size of R, the rate of release of D-galactose from the enzyme *increased* at low [R-CH_2OH]. This finding cannot be reconciled with the reaction mechanism described in Scheme 1. Therefore, the results provide strong support in favor of the suggestion that, as stated above, part of the observed increase in k_{cat} (oNP) of D-galactoside hydrolysis is brought about by the binding of primary alcohols remote from the site where actual fission of the glycosidic bond occurs, thereby stabilizing a more reactive conformation of the enzyme to increase the rate of the galactosylation step (k_3), or enhancing the rates of dissociation of the leaving group and degalactosylation to water.

Substituent Effects in Reactions of Galactosylated Enzyme Intermediates with Ethanol and Derivatives Thereof—

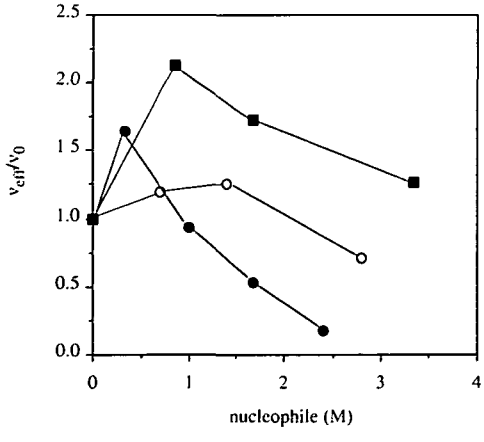


Fig. 2. **Rate of formation of D-galactose during the reaction of SsβGly with oNPGal in the absence and presence of external nucleophiles.** The reactions were carried out at 80°C and pH 6.5. The symbols denote 1,2-ethane-diol (full squares), 2,2,2-trifluoroethanol (open circles), and 1-propanol (full circles). v_0 and v_{eff} are the rates of formation of D-galactose in the absence and presence of added nucleophile, respectively.

v_{oNP}/v_{Gal} profiles were recorded for the reactions of SsβGly and CelB with oNPGal as the substrate in the presence of increasing concentrations of alcohols of the form R-CH_2OH, where R is H, a methyl group or a methyl group in which one or more hydrogens is replaced by an electron-withdrawing substituent. The substituents cause a decrease in pK_a of the primary OH group, relative to the OH group in ethanol, by between 0.5 and 3.6 pK units (Table II). The structural variations across the series of primary alcohols are, however, small so that differential effects of the substituents on the binding of R should be minimal. Therefore, it was expected that k_{Nu}/k_{water} determined from v_{oNP}/v_{Gal} profiles for the enzymatic reaction with oNPGal (see Fig. 3) should increase with decreasing pK_a of the alcohol. A Brønsted plot of log(k_{Nu}/k_{water}) *vs.* pK_a of the alcohol might then give a slope coefficient (β_{nuc}) that could be compared with related β_{nuc} values for galactosyl transfer reactions catalyzed by other mesophilic β-glycosidases (7).

For reactions catalyzed by SsβGly, a significant increase in v_{oNP}/v_{Gal} was observed in the presence of all alcohols tested. This increase was dependent on [R-CH_2-OH]. The resulting experimental curves of v_{oNP}/v_{Gal} *vs.* [R-CH_2-OH] were characterized in each case by a significant part in which v_{oNP}/v_{Gal} showed a linear dependence on the concentration of the intercepting alcohol (Fig. 3). Identification of the respective linear ranges in Fig. 3 again involved regression and statistical analyses. By contrast, only ethanol (R = CH_3) and methanol (R = H) appeared to function as acceptors of a D-galactose residue from the enzyme in the reaction catalyzed by CelB (see Table 1). In the presence of increasing concentrations of derivatives of ethanol, no significant changes in v_{oNP}/v_{Gal} were observed with CelB, compared to the value of v_{oNP}/v_{Gal} for the control reaction not containing an external nucleophile. An increase in k_{cat} (oNP) upon the addition of R-CH_2-OH was observed for reactions catalyzed by SsβGly but not CelB. Rate constant ratios for the partitioning of galactosylated SsβGly between reactions with R-CH_2OH and with water were obtained from Fig. 3 and are summarized in Table II. The data show that significant changes in the pK_a of R-CH_2OH have virtually no effect on k_{Nu}/k_{water}. A possible exception was the value of k_{Nu}/k_{water} for SsβGly-catalyzed galactosyl transfer to 2,2,2-trifluoro-ethanol, which was about 2.7 times the average value of k_{Nu}/k_{water} for the enzymatic galactosyl transfer to the other alcohols tested. However, there is good evidence that halomethyl groups are hydrophobic compared to the methyl and hydroxymethyl groups (*38*), and hydrophobic interactions have been shown to provide significant transition state-stabilization energy in the reaction of galactosylated SsβGly with primary alcohols. We conclude, therefore, that the pK_a of a simple primary alcohol is not expressed in this transition state so that a rather uniform picture is obtained pertaining to the reactivity of these alcohols towards galactosylated SsβGly. The pK_a effect may be almost completely hidden because of the contribution of general base catalysis to alcohol addition to the reaction intermediate on the one hand, and noncovalent enzyme/nucleophile interactions being predominant on the other hand.

Reaction of Galactosylated Enzymes with Azide—Figure 4 reports the dependence of v_{Glc}/v_{Gal} upon substrate concentration during the hydrolysis of lactose by SsβGly in the absence and presence of 1 M azide ion. Lactose was preferred to oNPGal in these experiments because high concentrations of azide ion were found to interfer with the determination of oNP absorbance. The profiles of v_{Glc}/v_{Gal} *vs.* [lactose] determined in the absence and presence of azide were identical within the limits of experimental error. Similar results were obtained with CelB (not shown). Therefore, this implies that like a number of other mesophilic β-retaining hydrolases (*8, 17*), the galactosylated enzyme

TABLE II. **Substituent effects on the transition state of the SsβGly-catalyzed galactosyl transfer to small primary alcohols.** The reactions were carried out at 80°C and pH 6.5 using 7.5 mM oNPGal as the substrate.

Acceptor alcohol	pK_a of alcohol[a]	k_{Nu}/k_{water} (M^{-1})
Ethanol	16.0	1.4
Methanol	15.1	0.8
1,2-Ethane-diol	15.1	1.4
2-Chloro-ethanol	14.3	1.4
2,2,2-Trifluoro-ethanol	12.4	3.4

[a]from Ref. *37*, determined at 25°C.

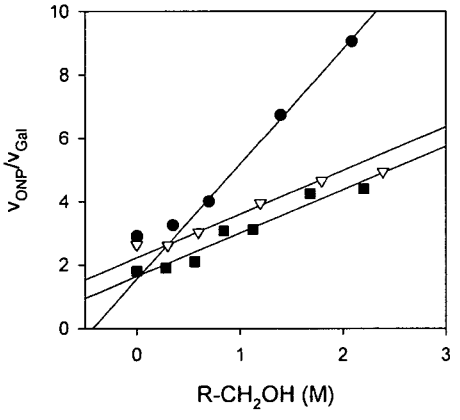


Fig. 3. **Transfer of D-galactose from the enzyme to derivatives of ethanol determined from the partitioning of the galactosylated SsβGly between reaction with the alcohol and water.** The constant concentration of oNPGal was 7.5 mM, and the reactions were carried out at 80°C and pH 6.5. Symbols denote 2,2,2-trifluoroethanol (circles), 2-chloro-ethanol (triangles), 1,2-ethane-diol (squares).

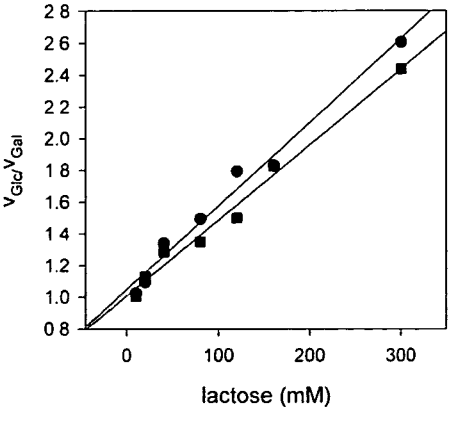


Fig. 4. **Partitioning of the galactosylated enzyme intermediate of SsβGly between reaction with the substrate lactose and water in the presence (squares) and absence (circles) of 1 M azide ion.** The reactions were carried out at 80°C and pH 6.5.

intermediates of recombinant wild-type SsβGly and CelB cannot react with the anionic nucleophile to yield glycosidic products. Charge screening provided by the active-site residues, particularly the carboxylate group functioning as a catalytic base, may be responsible. Using mutants of β-retaining glycosidases it was shown in kinetic studies that azide can become reactive towards the glycosylated enzyme intermediate upon replacement of the catalytic acid/base by alanine or glycine. The observed value of ≈14,000 M^{-1} for the partitioning of the galactosylated intermediate of *Escherichia coli* β-galactosidase (lacZ; Glu431Gly mutant) between reaction with azide and reaction with water was suggested to come close to expressing the intrinsic reactivity of azide towards reaction with carbocations (*34*).

Reaction of Galactosylated Enzymes with Phenol—Aryl-β-glycosides are good substrates of hydrolysis reactions catalyzed by SsβGly and CelB (*1–6, 24*). Therefore, we expected to observe catalysis by both thermostable β-glycosidases to the transfer of a galactosyl residue from the enzyme to phenol as the acceptor, or, in other words, a significant increase in v_{oNP}/v_{Gal} dependent on the concentration of phenol added to the reaction mixture. (Note that we could not use oNP as a possible acceptor in these experiments because the absorbance of oNP strongly interferes with the stectrophotometric assay for the quantification of D-galactose.) In marked contrast to our expectations, v_{oNP}/v_{Gal} decreased with increasing phenol concentration during the reactions of SsβGly and CelB with oNPGal as the substrate (Fig. 5, panel a). This implies that the extent of galactosyl transfer to acceptors (oNGal, phenol) other than water is smaller in the presence of phenol compared with the absence of phenol. In addition, phenol had a clear effect on the steady-state rate of the enzyme-catalyzed release of oNP from oNPGal (Fig. 5, panel b). At low phenol concentration up to 5 mM, a slight increase in k_{cat} was seen, whereas inhibition occurred at concentrations of phenol greater than 5 mM. Similar results have been obtained using lactose as the substrate and oNP as the nucleophile (data not shown). If phenolic alcohols bind nonproductively but tightly to the galactosylated enzyme intermediates, inhibition of the rate of substrate turnover would be expected, and that was observed. However, the experimentally determined decrease in v_{oNP}/v_{Gal} upon the addition of phenol requires that water be able to react with the galactosylated enzyme in the complex of E-Gal and phenol. To determine whether phenol is bound in a manner that allows nucleophiles other than water to react with the galactosylated enzyme intermediate, D-glucose was used. The rate constant ratio k_{Glc}/k_{water} is 34 and 17 M^{-1} for SsβGly and CelB, respectively, indicating that D-glucose can efficiently trap the galactosylated intermediate (*24*). The change of v_{oNP}/v_{Gal} upon increasing the concentration of phenol in the presence of 200 mM glucose is contrasted in Fig. 5 (panel a) with the dependence of v_{oNP}/v_{Gal} on [phenol] in the absence of D-glucose. It is clear from the data that D-glucose has no significant effect on v_{oNP}/v_{Gal}. Consequently, D-glucose did not react with E-Gal to yield transferase products when phenol was bound. Otherwise, no or at least a smaller decrease in v_{oNP}/v_{Gal} was expected to occur when the concentration of phenol was increased in the presence of D-glucose, compared to the control reaction that did not contain D-glucose.

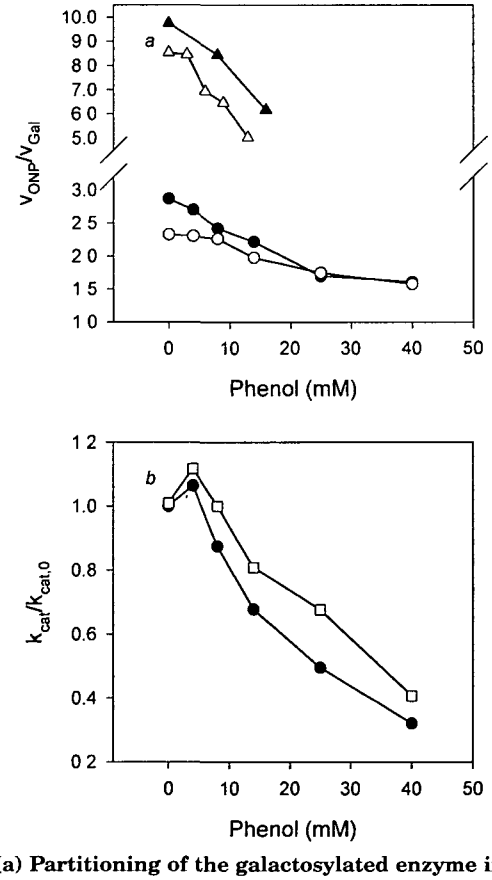


Fig. 5. **(a) Partitioning of the galactosylated enzyme intermediates of SsβGly (closed symbols) and CelB (open symbols) between reaction with nucleophilic acceptors and water in the presence of increasing concentrations of phenol.** Shown are the partitioning ratios in the absence (circles) and presence (triangles) of 200 mM D-glucose. **(b) Effect of phenol on the turnover number for reaction with oNPGal in which k_{cat} and $k_{cat,0}$ are the turnover numbers in the presence and absence of phenol, respectively.** The reactions were carried out at 80°C and pH 6.5 using a constant concentration of oNPGal of 7.5 mM.

CONCLUSIONS

A number of experimental observations reported herein and in previous papers (*4–6, 22, 24*) indicate that SsβGly and CelB have extended binding sites for the leaving group/nucleophile. The results of this work support the notion that the nucleophile binding sites of both β-glycosidases may be composed of several, but at least two subsites proximal to the catalytic site, sugar-binding subsite –1 (see Scheme 2). This implies that the interactions between the glycosylated enzyme intermediates and nucleophiles may be complex and arguably involve productive and nonproductive modes of binding of the potential glycosyl acceptors. Kinetic analyses of the reactions of galactosylated SsβGly and CelB with neutral and anionic nucleophiles provide a useful characterization of the specificities of the enzymes for transgalactosylation, as summarized below. They also show clearly that the intrinsic reactivity of primary alcohols is not detectably expressed in the transition state of galactosyl transfer catalyzed by the thermostable β-glycosidases, and that the very nucleophilic azide anion is an incompetent acceptor.

(i) The leaving group/nucleophile binding sites of both

enzymes provide hydrogen and hydrophobic bonding as well as aromatic interactions with potential glycosyl acceptors. Subsites +1 and +2 are hydrophobic and appear to differ with regard to their bonding capabilities with the leaving group/nucleophile.

(ii) Both enzymes hydrolyze oligosaccharide substrates using an exo-type mechanism of action starting from the non-reducing end of the saccharide (*4–6*). Noncovalent enzyme/substrate interactions at subsites adjacent to subsite +1 have been shown to contribute to the catalytic efficiency of SsβGly for the hydrolysis of cello-oligosaccharides (*5*). Our data reporting the specificities of SsβGly and CelB for transferring D-galactose from the enzyme to D-glucose and hydrophobic primary alcohols (*24, 26*; this work) support a mechanism of nucleophile recognition by galactosylated enzyme intermediates in which interactions with sugars, probably mediated through hydrogen bonds, are provided mainly by subsite +1.

(iii) At 80°C there is a large 30 to 40-fold difference in the rate constant ratios for the transfer of a galactosyl residue to oNPGal, k_{oNPGal}/k_{water}, and lactose, k_{Lac}/k_{water} (*24*). This implies that interactions with the phenolic aglycon of oNPGal at subsite +2 of the nucleophile-binding site contribute, relative to the interactions with 4-*O*-D-glucose of lactose, approx. 10 to 11 kJ/mol [$\Delta\Delta G^{\ddagger} = RT \ln (k_{oNPGal}/k_{Lac})$] to transition state stabilization in the reaction of E-Gal with β-D-galactopyranosides. Accordingly, the binding of a phenolic alcohol with E-Gal could arguably take place at subsite +1 or subsite +2, or both at the same time. If it occur-red at subsite +2, it would certainly be nonproductive pertaining to the transfer of a D-galactose residue to this alcohol; it would inhibit the binding of other nucleophiles, such as oNPGal, that require interactions at subsites +1 and +2 for binding; it might be responsible for increasing k_5, the rate constant for the dissociation of the leaving group and the galactosylated enzyme.

(iv) It is difficult to determine unambiguously whether the binding of phenol at subsite +2 is inhibitory to the binding or the reaction of nucleophiles that interact with subsite +1, such as D-glucose. In addition, it is not possible to decide whether a certain mode of binding of phenolic alcohols at subsite +1, which would have to be predominantly nonproductive pertaining to galactosyl transfer, or subsite +2 is required to allow water to react with E-Gal. However, preferential binding of phenolic alcohols at subsite +2 would seem in agreement with the failure to observe *any* net galactosyl transfer to phenol under the conditions used, as reflected by the v_{oNP}/v_{Gal} ratio in panel (a) of Fig. 5.

(v) The data suggest that carbohydrate synthesis *via* transglycosylation catalyzed by SsβGly and CelB will be most efficient when the glycosyl acceptor is another glycoside that binds to subsites +1 and +2. For the enzymatic production of oligosaccharides or non-natural glycosides it will now be very interesting to determine the kinetic stability of transglycoslation products that contain two or more glycosidic linkages.

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