Trp-999 of β-Galactosidase (*Escherichia coli*) Is a Key Residue for Binding, Catalysis, and Synthesis of Allolactose, the Natural *Lac* Operon Inducer†

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Received October 25, 2002; Revised Manuscript Received December 2, 2002

ABSTRACT: Trp-999 is a key residue for the action of β-galactosidases (*Escherichia coli*). Several site specific substitutions (Phe, Gly, Tyr, Leu) for Trp-999 were made. Each substitution caused greatly decreased affinities for substrates and inhibitors that bind in the "shallow" mode, while the affinities of inhibitors that bind in the "deep" mode were not decreased nearly as much. This shows that Trp-999 is important for binding in the shallow mode. The residue is also very important for binding glucose to galactosyl-β-galactosidase (as a transgalactosidic acceptor). Substitution greatly diminished the affinity for glucose. Substitutions also changed the activation thermodynamics and, subsequently, the rates of the catalytic reactions. The enthalpies of activation of the glycolytic bond cleavage step (galactosylation, k_2) became less favorable while the entropies of activation of that step became more favorable as a result of the substitutions. Differing magnitudes of these enthalpic and entropic effects with ONPG as compared to PNPG caused the k_2 values for ONPG to decrease but to increase for PNPG. The enthalpies of activation for the common hydrolytic step (degalactosylation, k_3) increased while the entropies of activation for this step did not change much. As a result, k_3 became small and rate determining for each substituted enzyme. The substitutions caused the rate constant (k_4) of the transgalactosidic acceptor reactions with glucose (for the formation of allolactose) to become much larger and of the same order of magnitude as the normally large rate constants for transgalactosidic acceptor reactions with small alcohols. This is probably because glucose can approach with less restriction in the absence of Trp-999. However, since glucose binds very poorly to the galactosyl-β-galactosidases with substitutions for Trp-999, the proportion of lactose molecules converted to allolactose is small. Thus, Trp-999 is also important for ensuring that an appropriate proportion of lactose is converted to allolactose.

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from *Escherichia coli* catalyzes hydrolytic and transgalactosidic reactions with β-D-galactopyranosides (*1*). The enzyme is a homotetramer. Each identical monomer of 1023 amino acid residues (*2*, *3*) functions independently. Mg^{2+} or Mn^{2+} and Na^+ or K^+ are required for full catalytic efficiency (*4*, *5*). The active site is in a deep pocket within a distorted "TIM" barrel (*6*, *7*). Free β-galactosidase has significant specificity for D-galactose and galactosides (*8*) but poor affinity for D-glucose (*9*). The affinity for glucose increases substantially after the glycosidic bond of the substrate is broken (*9*) (the dissociation constant of glucose bound to the free form of wild type β-galactosidase is about 600 mM (*8*) while the dissociation constant for glucose bound to galactosyl-β-galactosidase is 17 mM (*9*)). Free β-galactosidase has good affinity for hydrophobic groups. Galactosides with aromatic or hydrocarbon aglycones bind tightly to the free enzyme.

Proteins that bind sugars often have one or more Trp at their binding sites (*10*−*12*). For example, several Trp in the cellulose binding domain of xylanase (*Pseudomonas fluoresens*) were replaced individually by Ala (*13*). Each substituted enzyme bound substrate much more poorly than did the wild type. Also, the Trp in some lectins are important for binding sugars (*14*, *15*). The affinity between sugars and Trp may derive from interactions between the slightly positively charged aliphatic hydrogens of the sugars and the π electrons of Trp.

The Trp at position 999 of the β-galactosidase of *E. coli* is conserved in many related β-galactosidases (*3*, *16*−*26*). Structural studies indicate that the aromatic side chain of Trp-999 (part of the fifth domain of the subunit) is one component of the binding site (*6*, *7*). Juers et al. (*27*) showed that substrates and inhibitors that have an aglycone attached bind to free β-galactosidase in what they refer to as a "shallow" mode. In this mode, these molecules are located on top and parallel to Trp-999. Covalently bound (to Glu-537) galactose intermediates, transition state analogues, and galactose (plus other monosaccharoses tested) were, however, found to have moved further into the active site and are positioned away from Trp-999 and close to Trp-568 in a "deep" binding mode.

† This work was supported in part by the Natural Science and Engineering Research Council (Canada).

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10.1021/bi0270642 CCC: $25.00
Published on Web 01/22/2003

The binding of glucose to galactosyl-β-galactosidase is known to be important for the transgalactosidic reactions of the enzyme and particularly for the intramolecular conversion of lactose to allolactose (6-*O*-D-galactopyranosyl-β-D-(1−6)-glucopyranose), the true inducer (*28*) of the *lac* operon. Kinetic studies (*1*) have shown that the direct intramolecular formation of allolactose by wild type β-galactosidase requires that some of the glucoses cleaved from lactose remain bound long enough so that their C6 hydroxyl groups can react with the galactosyl enzyme intermediate to form allolactose. Free glucose also binds and reacts to form allolactose (indirect intermolecular allolactose synthesis), but at initial times of reaction with lactose, free glucose will be present in the surrounding solution in such a dilute concentration that not much would be present to bind. In addition, under physiological conditions, significant concentrations would rarely be present because the glucose would be metabolized. Thus, if allolactose is to be formed, it is important that a significant portion of the glucose cleaved from lactose remain bound (*1*). Other studies have shown that the C6 hydroxyl of glucose reacts relatively slowly with the galactosyl moiety of wild-type galactosyl-β-galactosidase compared to the reaction rate with small alcohols even though the nucleophilicity is in about the same range (*9*).

The present work shows that Trp-999 is a key residue at the active site of β-galactosidase. It is important for shallow mode binding to the free enzyme. It is especially important for binding glucose to the galactosylated form of β-galactosidase. In addition, the study shows that substitutions for Trp-999 cause large decreases of the degalactosylation rate. On the other hand, they caused very significant increases in the rates of the transgalactosidic reactions with glucose. Despite the large increases of the transgalactosidic reaction rates, the proportion of lactose that is converted to allolactose is small because of the poor binding of glucose to the substituted galactosyl-β-galactosidases.

EXPERIMENTAL PROCEDURES

Reagents and Buffers. Lactose, D-galactose, L-arabinose, D-glucose, D-xylose, D-lyxose, D-mannose, L-ribose, D-galactonolactone, 2-amino-D-galactose, TES[1], ONPG, PNPG, PETG, and IPTG were from Sigma. Complete protease tablets and ampicillin were from Boehringer Mannheim. Other reagents were from Fisher or similar sources. The purest grades of each of the chemicals used were purchased. DEAE BioGel Agarose was from BioRad.

"TES assay buffer" (pH 7.0 at 25 °C) was made with 30 mM TES, 145 mM NaCl, and 1 mM $MgSO_4$. For assays at temperatures other than 25 °C, the pH was carefully adjusted (at 25 °C) such that the pH was 7.0 at each assay temperature (using the temperature coefficient for TES).

Bacterial Growth. Substitutions for Trp-999 were made by methods described earlier (*29*). The substituted *lac Z* genes were on stable F′ episomes (single copies). The following strains were used (*29*):

E. coli S90c: ara, Δ(lacproB), thi, rpsL;

E. coli XA101:ara, Δ(lacproB), thi, rpsL, gyrA, metB, argE$^-$*, am, rpoB, supD*

E. coli XA102:ara, Δ(lacproB), thi, rpsL, gyrA, metB, argE$^-$*, am, rpoB, supE*

E. coli XA103:ara, Δ(lacproB), thi, rpsL, gyrA, metB, argE$^-$*, am, rpoB, supF*

E. coli XA105:ara, Δ(lacproB), thi, rpsL, gyrA, metB, argE$^-$*, am, rpoB, supG*

E. coli XA96:ara, Δ(lacproB), thi, rpsL, gyrA, metB, argE$^-$*, am, rpoB, supP*

These strains are F^- and have a deletion of *lacZ* in their genomes. The strains were transformed with F′ episomes encoding for the appropriate substituted β-galactosidase genes along with *lacY*, *lacA*, and *proAB*.

Liquid minimal medium consisted of KH_2PO_4 (13.6 g/L), $(NH_4)_2SO_4$ (2 g/L), $FeSO_4{\cdot}7H_2O$ (0.5 g/L), and $MgSO_4{\cdot}7H_2O$ (0.26 g/L) at pH 7.0. The medium was autoclaved (120 °C, 22 psi) for 20 min. Sterile solutions of glucose (final concentration, 200 g/L) and Met (final concentration, 2 mg/L) were added. Minimal agar plate medium (minimal medium with 1.5% agar) was autoclaved as above. Sterile solutions of glucose and Met (as above) were also added to the cooling agar just before pouring into Petri dishes.

The cells were initially grown on agar plates with minimal medium as described above but containing ampicillin. Single colonies were inoculated into 50 mL of LB media with ampicillin (50 mg/mL) and grown (150 rpm) at 37 °C overnight. An aliquot (5 mL) was used to inoculate each of ten 100 mL flasks, and these were grown for 8−10 h at 37 °C. Ten Fernbach flasks, each containing 1500 mL of media, were each inoculated with one 100 mL culture and grown for 18 h at 37 °C. The cells were harvested by centrifuging (4500*g*) for 20 min and stored at −70 °C until required.

SDS−PAGE. All SDS−PAGE of proteins was done using a PhastGel System (Pharmacia) with 8−25% acrylamide gradients (G8−25) according to the manufacturer's instructions. The gels were stained using Coomassie Blue.

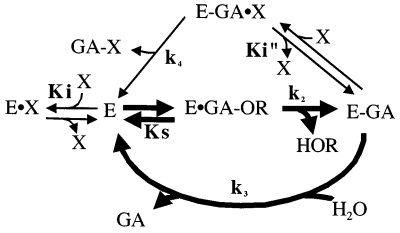
Purification. The enzymes were purified as previously described (*30*) except that the final concentrated solution was eluted through serially connected Superose 12 and Superose 6 FPLC columns (Pharmacia). Purity was assessed by specific activity and SDS−PAGE. Only fractions with enzyme that was at least 97% pure (by densitometer scans of the gels) were used for kinetic analyses.

Assays. Appropriately diluted enzyme (50 μL) was added to 950 μL of TES assay buffer containing either ONPG or PNPG. Routine assays were carried out at 6 mM ONPG or PNPG. The rates of reaction at 12 different substrate concentrations (six below and six above the K_m) were determined to obtain the k_{cat}, K_m, app k_{cat}, app K_m, and K_i values. The highest substrate concentrations were at least 40-fold larger than the lowest. The absorbency changes were measured at 420 nm (25 °C) with a Shimadzu UV 2101PC spectrophotometer and using Shimadzu Kinetics software (version 2.0). The amounts of nitrophenolate ion were calculated using the following extinction coefficients (pH 7): ϵ_{420} oNP = 6.67 mM^{-1} cm^{-1}; ϵ_{420} pNP = 2.65 mM^{-1} cm^{-1}.

Kinetic Data Analysis. Reactions that occur with nitrophenol substrates are shown in Scheme 1. The thick arrows represent the reactions that occur without inhibitor/acceptor.

[1] Abbreviations: EDTA, ethylenediamine tetraacetic acid; HMDS, hexamethyldisilazane; IPTG, isopropylthio -β-D-galactopyranoside; ONPG, *ortho*-nitrophenyl-β-D-galactopyranoside; PNPG, *para*-nitrophenyl-β-D-galactopyranoside; PETG, phenylethylthio-β-D-galactopyranoside; TES, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TMCS, trimethylchlorsilane; Tris, Tris(hydroxymethyl)aminomethane.

Scheme 1: Postulated β-Galactosidase Mechanism[a]



[a] E, β-galactosidase; GA-OR, substrate; GA, galactose; HOR, aglycone; X, inhibitor/acceptor; GA-X, adduct. The dots indicate that complexes exist between the substrate and the enzyme. The hyphens indicate that a covalent bond is present. The thick arrows represent the reaction without inhibitor/acceptor (X). K_s is the dissociation constant of the enzyme·substrate complex. The step with the rate constant k_2 is called "galactosylation" while the step with k_3 is called "degalactosylation". The thin arrows represent the additional reactions that occur in the presence of inhibitor/acceptor. The inhibitor/acceptor binds to both the free and the "galactosylated" enzyme. The dissociation constant for the interaction of a competitive inhibitor with free enzyme is K_i. The dissociation constant for binding to the "galactosylated" enzyme is K_i''. Acceptors react (k_4) to form adducts.

The additional reactions that occur in the presence of inhibitor/acceptor are shown with thin arrows. Molecules that act as competitive inhibitors usually also act as acceptors (designated as X in the scheme) to form galactosyl−acceptor adducts (GA−X) (*9*, *31*). This acceptor reaction must be taken into account when determining K_i. This was done using eq 1 (*31*). The k_{cat} and K_m values at several concentrations

$$\left(\frac{K_m}{k_{cat}}\right)_{apparent} = \frac{K_m}{k_{cat}}\left[1 + \frac{[X]}{K_i}\right] \quad (1)$$

of inhibitor/acceptor ([X]) were determined by linear regression of Eadie-Hofstee plots with Excel. The K_i values determined at the different concentrations (using eq 1) were averaged. K_m/k_{cat} is the ratio of K_m to k_{cat} obtained without inhibitor/acceptor while $(K_m/k_{cat})_{apparent}$ is the ratio with inhibitor/acceptor.

One can obtain information about rate determining steps by the effects of acceptors (see Scheme 1) on the rates. If the k_4 value of an acceptor is greater than k_3 and if k_3 is smaller than k_2, the apparent k_{cat} of the reaction in the presence of increasing acceptor concentrations will increase (*31*) to a maximum equal to $k_2k_4/(k_2 + k_4)$. If the rate increases more than 10-fold upon addition of acceptor, k_2 and k_4 are each at least 10× as large as k_3, and therefore k_{cat} (without the acceptor) is essentially equivalent to k_3. If k_4 for an acceptor is larger than k_3 but k_2 is rate limiting, there will be no change in the rate upon addition of acceptor.

Equation 2 describes how apparent k_{cat} changes as a function of the acceptor concentration (*31*). If k_2 and k_4 are

$$\text{apparent } k_{cat} = \frac{k_2k_3 + k_2k_4\dfrac{[X]}{K_i''}}{k_2 + k_3 + (k_2 + k_4)\dfrac{[X]}{K_i''}} \quad (2)$$

larger than k_3 and if the concentrations of acceptor ([X]) are of the same order of magnitude as the K_i'' value, a hyperbolic plot (starting at the k_{cat} value on the vertical axis) should be obtained if apparent k_{cat} is plotted as a function of [X]. However, straight lines were obtained for three of the four enzymes even at the highest concentrations of acceptor (up to 2 M) that could be tested (limited by the solubility of the acceptor). Theoretical analysis of eq 2 indicates that linear lines are obtained if [X] is much smaller than K_i''. The lines obtained then have slopes approximately equal to k_4/K_i'' (the slopes would only be truly equal to k_4/K_i'' at acceptor concentrations infinitely smaller than K_i''). These lines are equivalent to the pseudo first-order straight line portions of Michaelis−Menten plots (V_o vs $[S]_o$) that occur at substrate concentrations very much smaller than the K_m (in that case the slopes are equal to V_m/K_m).

For one of the substitutions (W999F-β-galactosidase), hyperbolic plots were obtained when the apparent k_{cat} values were plotted versus [X] (eq 2). In that case, eq 3, a rearrangement (*31*) of eq 2, was used for analysis. The k_{cat} is equal to $k_2k_3/(k_2 + k_3)$.

$$\text{apparent } k_{cat} = \frac{\text{apparent } k_{cat} - k_{cat}}{[X]} \cdot \frac{(k_2 + k_3)}{(k_2 + k_4)} \cdot K_i'' + \frac{k_2k_4}{(k_2 + k_4)} \quad (3)$$

Determination of Rate Constants and Energetics. Ayala and Di Cera (*32*) have described a simple method for the determination of the values of the two individual rate constants and of the activation energies of the reaction steps of serine proteases. Since β-galactosidase is a hydrolytic enzyme that has a two-step mechanism kinetically similar to that of serine proteases, the method was adapted for this study. We utilized the data to determine the enthalpies and entropies of activation of the galactosylation (k_2) and degalactosylation (k_3) steps. We were able to do this for both steps because the activation energetics were such that bent Arrhenius lines were obtained when the ln k_{cat} values were plotted versus $1/T$ (two examples are shown in Figure 3). Galactosylation (k_2) was the rate determining step at the lower temperatures, while degalactosylation (k_3) was rate determining at higher temperatures. Lines of the dependence of ln k_3 and of ln k_2 on $1/T$ were obtained by regression analysis of the data. The k_2 and k_3 values for each enzyme at 25 °C were obtained from these lines. K_s values (25 °C) were then calculated using the k_2 and k_3 values (25 °C) and the K_m values $\{K_m = K_sk_3/(k_2 + k_3)\}$ at 25 °C. The enthalpies and entropies of activation of the two reaction steps (galactosylation (k_2) and degalactosylation (k_3)) of the β-galactosidases were also calculated from the plots (*33*).

Gas Liquid Chromatography. The amount of lactose that was directly converted to allolactose compared to the amount that was hydrolyzed to galactose and glucose was determined by gas liquid chromatography. Incubations were carried out in small glass test tubes at 25 °C. A high lactose concentration (100 mM) in TES assay buffer was used so that the lactose would outcompete the small amount of allolactose formed and minimize the hydrolysis of the allolactose. This is important because allolactose is also a hydrolytic substrate of β-galactosidase, and a false hydrolytic/transgalactosidic product ratio would be obtained if allolactose hydrolysis was not inhibited. Despite the high lactose concentration, there was some slow hydrolysis of the allolactose. It was, therefore, also necessary to extrapolate the ratios of the rates of the transgalactosidic to the hydrolytic reactions back to zero time to account for this slow hydrolysis.

Table 1: Kinetic Constant Values for ONPG and PNPG[a]

	k_{cat} (ONPG)	k_{cat} (PNPG)	K_m (ONPG)	K_m (PNPG)
wild type	600 ± 11	90 ± 1	0.12 ± .01	0.040 ± .002
W999F	54 ± 5	67 ± 2	0.26 ± 0.02	0.43 ± 0.03
W999G	48 ± 3	52 ± 4	0.31 ± 0.02	0.52 ± 0.04
W999Y	56 ± 5	65 ± 4	2.1 ± 0.2	1.6 ± 0.1
W999L	20 ± 4	28 ± 5	3.3 ± 0.1	2.1 ± 0.1

[a] The units are k_{cat}, s^{-1}; K_m, mM.

Gas liquid chromatographic analysis of sugars requires that the sugars be silylated. To do this, Eppendorf tubes (1.5 mL) were immersed in liquid N_2 and internal standard (100 μL of 2 mM i-inositol) was added to each tube. A zero time sample (100 μL) of the reaction mixture was added to one of the tubes in the liquid N_2. Enzyme was then added to the glass reaction tubes (25 °C) and 100 μL samples of the reaction mixtures were removed at various times and added to the Eppendorf tubes that were immersed in the N_2. The aliquots froze immediately. This served to stop the reaction. The tubes were covered with lids having holes drilled in them to allow for lyophilization. When all of the samples were collected, the tubes were removed from the liquid N_2 and quickly placed into a lyophilizing flask for lyophilization to remove the water that would interfere with the silylation. After lyophilization, dimethyl formamide (500 μL) was added to each tube as a solvent. Then, 200 μL of HMDS and 100 μL of TMCS were added to bring about the silylation and lids without holes were substituted. The samples were vortexed for 30 s and placed into a 70 °C oven for 30 min. They were vortexed again for 15 s and again incubated at 70 °C for 30 min. The reaction mixtures separated into two liquid phases. The silylated sugars were in the top phase. Small aliquots were injected onto a stainless steel column (0.6 cm × 2 m) packed with Chromasorb W beads coated with 3% OV-101 (Chromatographic Specialties). Helium was the carrier gas (40 mL/min.). A temperature program was used. Detection was by flame ionization. The ratios of the sugar peak areas to the inositol peak areas were compared to standards to obtain the concentrations.

RESULTS

K_m and k_{cat} Values. Table 1 lists the k_{cat} and K_m values obtained for the wild type and substituted enzymes. The ONPG and PNPG k_{cat} values all decreased and became approximately equal for each individual substituted enzyme. The k_{cat} values for W999F-, W999G-, and W999Y-β-galactosidases were all approximately equal while the k_{cat} values for W999L-β-galactosidase were about 2−3-fold lower than for the other three substituted enzymes. The K_m values for ONPG and PNPG increased and were also approximately equal for each individual substituted enzyme. The K_m values increased as follows: wild type < W999F-β-galactosidase < W999G-β-galactosidase < W999Y-β-galactosidase < W999L-β-galactosidase. The K_m values for W999Y- and for W999L-β-galactosidase were substantially larger than for the other two substituted enzymes.

Inhibition. Table 2 lists the competitive inhibition constants (K_i) for the wild type and substituted β-galactosidases. The substituted enzymes were inhibited more poorly (the K_i values became larger) by almost every inhibitor. However, the three β-galactopyranoside inhibitors (IPTG, PETG, and lactose) were especially poor inhibitors of the substituted enzymes compared to the monosaccharoses. The loss of inhibitory action was the greatest with PETG.

Table 2: The Competitive Inhibitor Constants (K_i − mM) for Various Inhibitors of the β-Galactosidases with Substitutions for Trp-999[a]

inhibitor	wild type	W999F	W999G	W999Y	W999L
IPTG	0.08	7.8 (*97*)	60 (*750*)	180 (*2250*)	335 (*4200*)
PETG	0.001	0.30 (*300*)	2.8 (*2800*)	6.8 (*6800*)	12 (*12000*)
lactose	1.3	160 (*114*)	177 (*126*)	730 (*521*)	1330 (*950*)
D-galactose	24	79 (*3.3*)	101 (*4.2*)	190 (*7.9*)	180 (*7.5*)
L-arabinose	190	300 (*1.6*)	507 (*2.7*)	790 (*4.2*)	960 (*5.1*)
D-glucose	600	3990 (*6.6*)	1860 (*3.1*)	2500 (*4.2*)	2400 (*4.0*)
D-xylose	730	1040 (*1.4*)	1800 (*2.5*)	420 (*0.6*)	2450 (*3.3*)
D-lyxose	80	200 (*2.5*)	145 (*1.8*)	820 (*10.2*)	250 (*3.1*)
D-mannose	520	750 (*1.4*)	1150 (*2.2*)	3700 (*7.1*)	1270 (*2.4*)
L-ribose	0.28	2.1 (*7.9*)	1.1 (*3.9*)	5.0 (*17.8*)	2.5 (*8.9*)
galactonolactone	1.0	0.8 (*0.8*)	2.3 (*2.3*)	27 (*27*)	19 (*19*)
2-NH-galactose	1.9	1.8 (*0.95*)	7.2 (*3.8*)	7.8 (*4.3*)	5.6 (*2.9*)

[a] The values in brackets (italics) represent the fold increases of the K_i values relative to wild type β-galactosidase. The top portion of the table (above the D-galactose row) shows the inhibitory effects of the three galactosides tested, while the bottom portion shows the inhibitory effects of monosaccharoses. The standard errors were less than 10% of the K_i values in each case.

Transgalactosidic Reactions with Acceptors. The rates of reaction with both ONPG and PNPG increased for each of the substituted enzymes upon addition of several different acceptors (data not shown). Two acceptors (D-glucose and 1,4-butanediol) were studied in detail. Glucose was studied because its action as an acceptor forms allolactose (*1*). The acceptor action of 1,4-butanediol was studied because preliminary results with several potential acceptors showed that this alcohol caused the greatest increases of the reaction rates. Control experiments showed that adducts with the acceptor were formed when the acceptors were added and that the amounts increased as the acceptor concentration increased. This indicates that the increases in activity were due to the transgalactosidic activity and not to some other activation effect. There was some concern that the high concentrations of acceptor that had to be used could affect the activity by solvent effects on the conformation. However, there were no detrimental effects since the k_{cat} values increased in each case. In addition, there were good fits of the data with the theoretical equations despite the use of these high concentrations.

The acceptor reaction data with W999F-β-galactosidase were plotted (Figure 1) according to eq 3. The intercepts were 610 and 570 s^{-1}, and the slopes were 670 and 840 mM for 1,4-butanediol and glucose, respectively. When equivalent data for the other three enzymes were plotted using eq 3, the data were bunched near to the horizontal axis of the plot. Crowding of this type occurs if the highest acceptor concentrations tested, which were very high (2 M) in these cases, are still significantly smaller than the K_i'' values (Scheme 1). The data describe very steep slopes, and it was impossible to fit accurate lines to the data since the points were bunched near the horizontal axis and fit only very small portions of the lines. Results such as these indicate that the reactions are essentially first order. The first-order nature was confirmed by the linear lines (Figure 2) obtained with

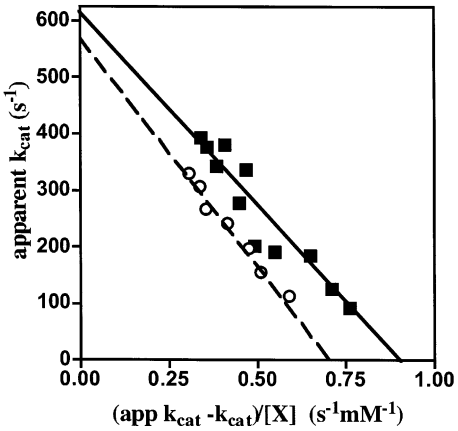


FIGURE 1: The variation of apparent k_{cat} values (s^{-1}) as functions of (app k_{cat} − k_{cat})/[acceptor] for W999F-β-galactosidase (eq 3). (■) Butanediol; (○) glucose. Acceptor concentration units are mM.

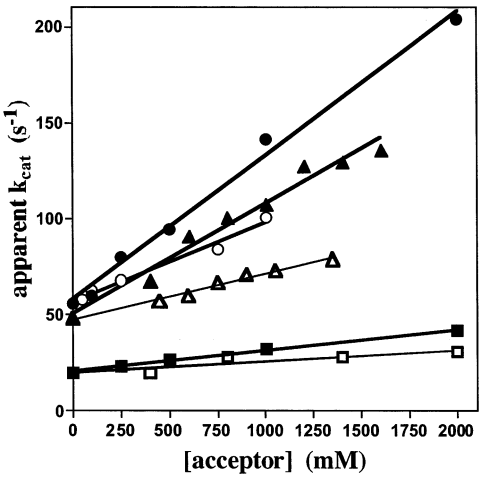


FIGURE 2: The apparent k_{cat} values (s^{-1}) plotted (eq 2) as functions of the 1,4-butanediol and the glucose concentrations (mM). W999L-β-galactosidase: (■) 1,4-butanediol, (□) glucose; W999G-β-galactosidase: (▲) 1,4-butanediol, (△) glucose; W999Y-β-galactosidase: (●) 1,4 -butanediol, (○) glucose.

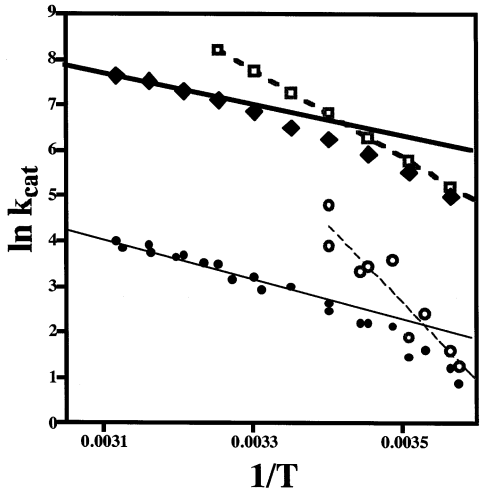


FIGURE 3: Arrhenius plots of the effects of temperature (K) on the ln k_{cat} values for wild type (thick lines, diamonds) and W999L-β-galactosidase (thin lines, circles). The filled-in points are the data obtained. The solid lines represent the calculated regression lines obtained for the k_3 values. The open shaded points are the values of k_2 calculated using the data and the regression lines for k_3. The dashed lines are the regression lines for k_2.

Table 3: Enthalpies ($\Delta H'^{\ddagger}$) and Entropies ($\Delta S'^{\ddagger}$) of Activation, the Rate Constants (s^{-1}), and the K_s Values for the Substituted and Wild Type β-Galactosidases[a]

	$\Delta H_2'^{\ddagger}$	$\Delta S_2'^{\ddagger}$	k_2 (25 °C)	$\Delta H_3'^{\ddagger}$	$\Delta S_3'^{\ddagger}$	k_3 (25 °C)	K_s (25 °C)
wt (ONPG)	77	0.075	1450	24	−0.109	1020	0.29
wt (PNPG)	82	0.069	90	24	−0.109	1020	0.040
W999F (ONPG)	124	0.226	610	33	−0.099	69	2.6
W999F (PNPG)	129	0.241	640	34	−0.097	59	5.1
W999G (ONPG)	118	0.206	720	32	−0.105	49	4.9
W999G (PNPG)	111	0.180	690	33	−0.101	50	7.7
W999Y (ONPG)	135	0.264	750	30	−0.114	44	48
W999L (ONPG)	139	0.265	180	34	−0.106	20	33

[a] The units are $\Delta H'^{\ddagger}$ kJ/mol; $\Delta S'^{\ddagger}$, kJ/degree·mol. The values with subscript "2" are for the galactosylation step while those with subscript "3" are for the degalactosylation step. K_s values (mM) were calculated from the K_m (Table 1) and the k_2 and k_3 values.

both 1,4-butanediol and glucose when the rates with W999G-, W999Y-, and W999L-β-galactosidase were plotted as functions of the acceptor concentrations (eq 2). The slopes (pseudo first-order rate constants approximately equal to k_4/K_i'') were small {W999G-β-galactosidase: 0.058 s^{-1} M^{-1} (butanediol), 0.024 s^{-1} M^{-1} (glucose); W999Y-β-galactosidase: 0.075 s^{-1} M^{-1} (butanediol), 0.042 s^{-1} M^{-1} (glucose); W999L-β-galactosidase: 0.011 sec^{-1} M^{-1} (butanediol), 0.006 s^{-1} M^{-1} (glucose)}. In each case, the slope was steeper with 1,4-butanediol than with glucose.

Intramolecular transgalactosidic activity (direct allolactose formation from lactose (*1*)) was studied by gas−liquid chromatography. It was found that 52 ± 3% of the product with wild type β-galactosidase was allolactose (the other products were galactose and glucose); 19 ± 5% with W999F-β-galactosidase; 5 ± 2% with W999L-β-galactosidase; <0.5% with W999Y-β-galactosidase; and allolactose could not be detected upon reaction with W999G-β-galactosidase.

Rate Constants and Activation Energetics. Figure 3 shows the Arrhenius plots obtained for the k_{cat} values of wild type and W999L-β-galactosidase with ONPG. The plots for the other enzymes and with PNPG were similar to these and similar curved lines were observed. The regression lines for k_2 and k_3 calculated from the data are shown for the two enzymes. The theoretical points for k_2 are also shown. The rate constants (25 °C) and estimates of the enthalpies and entropies of activation obtained from these plots and from similar plots for the other three substituted enzymes are given in Table 3. Galactosylation rates (k_2) with ONPG decreased by a small factor as a result of the substitutions but increased with PNPG. The k_3 values (degalactosylation) all decreased significantly. The k_3 values were smaller than the k_2 values (at least 14×) for each substituted enzyme. The enthalpies of activation of the galactosylation step ($\Delta H_2^{\ddagger}$) increased as a result of each substitution while the entropies of activation ($\Delta S_2^{\ddagger}$), which are positive values with wild type β-galactosidase, became significantly more positive. The enthalpies of activation of the degalactosylation step ($\Delta H_3^{\ddagger}$) also increased. However, the negative entropies of activation ($\Delta S_3^{\ddagger}$) remained approximately the same upon substitution.

The K_m values listed in Table 1 and the values of k_2 and k_3 listed in Table 3 were substituted into the equation for K_m ($k_3K_s/(k_2 + k_3)$) and K_s values were calculated. These are also shown in Table 3. The K_s values for the two

substrates were roughly equal for W999F- and W999G-β-galactosidase and were much larger than for wild-type enzyme. The K_s values of W999Y- and W999L-β-galactosidase (with ONPG) were particularly large.

DISCUSSION

General. Each substituted β-galactosidase eluted at a similar position as wild type on both the ion-exchange and size-exclusion columns. This shows that the gross physical properties of the substituted β-galactosidases are similar to those of wild type. In particular, size exclusion elution showed that the tetrameric forms of the enzymes were preserved. Each substituted enzyme was stable for several hours at 25 °C and could be stored for several months at 4 °C with only slow activity loss.

Binding to free β-Galactosidase. The K_s values (ONPG and PNPG, Table 3) of the substituted enzymes were much larger than those of wild type β-galactosidase. The K_i values of the galactosides (IPTG, PETG, lactose) also increased significantly. The K_i values of the monosaccharoses changed by much smaller amounts. For example, the inhibitory effects of D-galactose varied only 3.3−7.9-fold while the inhibition of W999F-, W999G-, W999Y-, and W999L-β-galactosidase by IPTG was decreased by 97-, 750-, 2250-, and 4200-fold, respectively. Structural studies have shown that monosaccharoses (e.g., D-galactose) have little contact with Trp-999 and bind in the "deep" mode of β-galactosidase while the substrates and other galactosides (e.g., IPTG) that bind in the "shallow" mode are positioned directly over Trp-999 (*27*). These results show that the effects of the substitutions on galactoside binding were much greater than the effects of the substitutions on monosaccharose binding. This, therefore, shows that Trp-999 is very important for binding in the shallow but much less important for binding in the deep mode of free β-galactosidase. The similarity of the K_s values for ONPG and PNPG for each individual enzyme are most likely due to a lack of discrimination between these two substrates because Trp-999 is absent. As expected, substitutions for Trp-999 caused the greatest losses of inhibition with an aromatic aglycone (PETG), next with an aliphatic aglycone (IPTG) and the least with lactose.

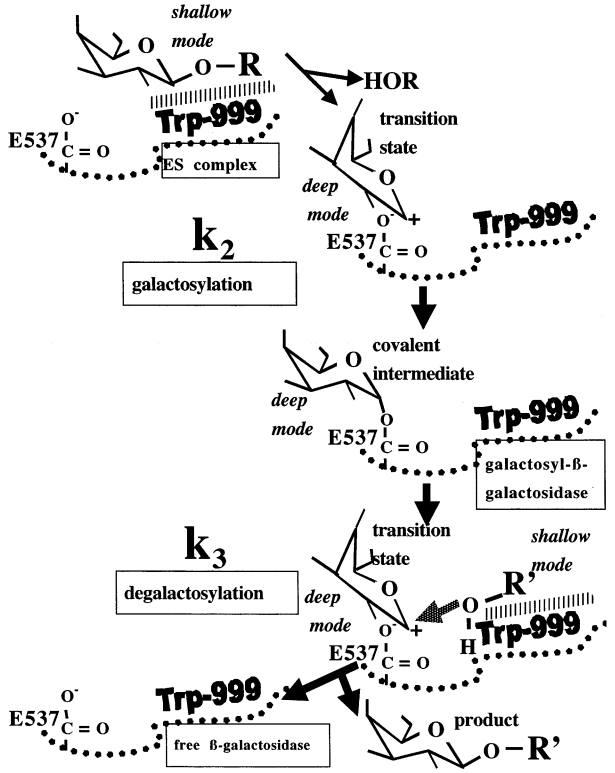
The increases of the K_i values (losses of the inhibitory effect) of the galactosides (IPTG, PETG, and lactose) and the increases in the K_s values of the substrates (Table 3) were the smallest for W999F-β-galactosidase and increased upon substitution by Gly, Tyr, and Leu (in that order). It is not difficult to understand why inhibition of the Phe substituted enzyme was affected the least since Phe, like Trp, is aromatic. It is, however, difficult to understand the affinity changes that result from the other substitutions. The Gly substitution did not result in as great a decrease of inhibition as did the substitutions by Tyr and Leu. It is possible that a cavity forms upon substitution by a Gly and if it collapses other side chains facilitate some interaction. One would think that W999Y-β-galactosidase would have similar affinities for aglycones as does W999F-β-galactosidase. The phenol hydroxyl could form an H-bond with the Asp-997 carboxyl, which is about 4 Å away, and pull the Tyr out of position. This could subsequently lead to an overall change in active site conformation. In addition, substitution of a Tyr would leave a gap because the Tyr would not reach as far into the active site as does the Trp since the α carbon of Trp-999 is positioned near to the opening of the active site. The galactoside inhibitors (PETG, IPTG, and lactose) inhibited W999L-β-galactosidase especially poorly. The side chain of Leu is often quite mobile and thus may cause poor binding.

The increases in K_i with D-glucose were small (Table 2) and much like those found for galactose and the other monosaccharoses. Glucose binds very poorly to free β-galactosidase but since the effects of substitution for Trp-999 on glucose inhibition are similar to the effects on galactose inhibition, the results imply that glucose binds to free β-galactosidase in the deep mode.

Galactosylation/degalactosylation. The ONPG and PNPG k_{cat} values ($k_2k_3/(k_2 + k_3)$) were similar in the case of each substituted enzyme. This is different from wild type β-galactosidase. The k_{cat} values of wild type for ONPG and PNPG differ by 6.7-fold because galactosylation is rate determining for PNPG (the k_2 value of PNPG is much smaller than that of ONPG). These results suggest that the common degalactosylation step (k_3) (see Scheme 1) is rate limiting with each substituted enzyme. Increases in activity were found for all of the substituted enzymes upon acceptor addition. This also indicates that k_3 is rate limiting. Figure 1 is a plot of the effects of acceptors on the apparent k_{cat} values (ONPG) with W999F-β-galactosidase (using eq 3). The k_{cat} value (ONPG) for W999F-β-galactosidase is low (54 s^{-1}) while the intercepts of the plots in Figure 1 are 610 and 570 s^{-1} for 1,4-butanediol and glucose, respectively. The intercepts of these plots are equal to $k_2k_4/(k_2 + k_4)$ (see eq 3). Simple mathematical reasoning shows that the values of k_2 and of k_4 have to be equal to or greater than these intercepts. Thus, k_2 (ONPG) is at least 610 s^{-1} and since the ONPG k_{cat} is much smaller (54 s^{-1}), it follows that k_3 must be rate determining and approximately equal to 54 s^{-1}. Similar results (data not shown) were also found for W999F-β-galactosidase with PNPG. The rates also increased upon addition of acceptor for the other substituted enzymes (see Figure 3) showing that k_3 is the slowest step for each of those enzymes. Temperature studies (Table 3) provided the k_2 and k_3 values (25 °C) and the enthalpies and entropies of activation of these two steps for the substituted enzymes. These provide firm evidence that the k_3 values are small compared to the k_2 values for each substituted enzyme and that the degalactosylation step is rate determining in each case. Relative to wild type, the k_3 values decreased 15-fold or more. On the other hand, the k_2 values decreased by small factors for ONPG but increased by small factors for PNPG.

The activation thermodynamics provide a rationale for the changes of the k_2 and k_3 values. The enthalpies of activation of the galactosylation (or glycosidic bond cleavage) reaction step (k_2) increased from about 80 kJ/mol for the wild type β-galactosidase to between 111 and 139 kJ/mol for the substituted β-galactosidases (Table 3). The enthalpy needed to achieve the transition state for the galactosylation step is probably used to "stretch or bend" the glycosidic bond and thereby aid in the formation of the first carbocation transition state (Scheme 2). The interaction with Trp-999 may facilitate the stretching or bending. In contrast to the less favorable enthalpies of activation, the entropies of activation of the galactosylation reactions (k_2), which are positive (favorable) for wild type β-galactosidase, became much more positive (favorable) upon substituting for Trp-999 (the values in-

Scheme 2: Outline of the Double Displacement Reaction Catalyzed by β-Galactosidase[a]



[a] The scheme illustrates the importance of Trp-999 for "shallow" mode binding of the substrate and of the acceptor (R′-O−H). The reaction involves the formation of carbocation transition states and a covalent α-galactosidic intermediate with Glu-537. The first part of the scheme (leading to the formation of the covalent intermediate) is called galactosylation (rate constant = k_2). The last part of the scheme is called degalactosylation (rate constant = k_3). The transition state and the covalent intermediate (as well as free D-galactose and other monosaccharoses) bind in the "deep" mode and away from Trp-999.

creased from about 0.070 kJ degree^{-1} mol^{-1} for the wild-type enzyme to between 0.180 and 0.265 kJ degree^{-1} mol^{-1} for the substituted β-galactosidases). It is possible that the active site components become more disordered during the formation of the transition state and that, without Trp-999, even more disorder transpires. However, it is more likely that the entropy is related to the process of forming the transition state. When the transition state for galactosylation (Scheme 2) is formed, the aglycone is released. This disordering is probably the reason that the entropy of activation of the galactosylation step of β-galactosidase is positive. The disorder would be greater for the substituted enzymes because of less interaction between the departing aglycone and Trp-999. The unfavorable enthalpy and the favorable entropy have opposite effects on the k_2 values with ONPG and PNPG. The k_2 values for ONPG decreased (relative to wild type) but increased for PNPG.

The decreases of the k_3 values at 25 °C derive mainly from less favorable enthalpies of activation. The $\Delta H_3^{\ddagger}$ values (Table 3) increased 25−40% relative to wild type while the $\Delta S_3^{\ddagger}$ values of the substituted enzymes (which are negative) were all <10% different from the wild type $\Delta S_3^{\ddagger}$. In most cases, the entropies were actually slightly more favorable (smaller negative values). Some stretching and bending effects are probably lost upon substitution for Trp-999 and thus cause the less favorable enthalpy of activation for degalactosylation (k_3).

Binding Affinities and Reactivity of Galactosyl-β-galactosidase. Wild-type galactosyl-β-galactosidase binds glucose well (K_i'' = 17 mM) (*9*) relative to free β-galactosidase (the K_i for glucose is about 600 mM). However, glucose was bound very poorly by the substituted galactosyl-β-galactosidases. The data for W999F-β-galactosidase are shown in Figure 2 (see eq 3). The slopes obtained were large (670 and 840 mM for 1,4-butanediol and glucose, respectively). It will be shown below that the k_4 values for both acceptors are much larger than k_3 for this enzyme and as a result, the K_i'' values are even greater than these large slopes. Indeed, if the k_2 and k_3 from Table 3 and the k_4 (glucose) values determined for W999F-β-galactosidase (see discussion below) are substituted into the equation for the slope (see eq 3), the K_i'' for glucose is about 6 M. This extremely large K_i'' value is meaningless quantitatively but shows that glucose forms extremely poor interactions with the galactosylated enzyme even when Trp-999 is substituted by Phe, an aromatic residue. For the other substituted enzymes, the K_i'' values (for glucose) are even higher. In those cases, plots of apparent k_{cat} versus [glucose] increased linearly up to 2 M (Figure 3). Plots of this type would only be linear if the acceptor concentration is much smaller than the K_i'' value. Preliminary structural data suggest that the glucose that is the acceptor for allolactose formation may be stacked on top of Trp-999 (*27*). The poor binding capabilities of glucose with the substituted enzymes most likely result from the loss of this stacking interaction with Trp-999.

Despite the poor binding of acceptor by the substituted β-galactosidases, transgalactosidic reactions do take place at high acceptor concentration and these reactions (k_4) occur very rapidly. This must result mainly from collisions with the acceptor that form short-lived E-GA·X complexes (see Scheme 1) that are highly reactive. In the case of W999F-β-galactosidase the k_2 value (ONPG) is 610 s^{-1} (Table 3). This value is equal to the intercept value (Figure 2) when 1,4-butanediol is the acceptor (610 s^{-1}). Since this intercept is equal to $k_2k_4/(k_2 + k_4)$, and since it has a value essentially equal to the value of k_2, it follows mathematically that the value of k_4 is much greater (at least 10×). Thus, k_4 is at least 6100 s^{-1}. Since the intercept (570 s^{-1}) of the glucose plot of Figure 2 (equal to $k_2k_4/(k_2 + k_4)$) is less than the value of k_2 for ONPG from Table 3 (610 s^{-1}) an estimate of the k_4 value for glucose could be calculated (8700 s^{-1}). This is very large compared to the k_4 value for glucose with wild type β-galactosidase (~380 s^{-1}) (*9*). The slopes of the apparent k_{cat} vs [glucose] plots for the other 3 substituted enzymes were linear up to 2 M and good estimates of the k_4 values could not be obtained. However, the linearity with glucose for these three enzymes indicates that the k_4 values (glucose) are also much higher than with wild type.

The value of k_4 attained by W999F-β-galactosidase with glucose is similar to the k_4 values obtained with small alcohols for wild type β-galactosidase (*9*). Small alcohols form very poor binding interactions with wild-type galactosyl-β-galactosidase but the reaction rates (k_4) of the short-lived E-GA·X complexes (Scheme 1) which are formed are very fast. Presumably small alcohols react rapidly because they can come into close contact for reaction since they are not restricted by strong interactions at the active site. The

nucleophilicity of the C6 primary hydroxyl of glucose should be of a similar order of magnitude to those of small primary alcohols and thus the potential reactivity (as quantitated by k_4) should be similar. It follows that when Trp has been replaced and glucose no longer has strong binding interactions, the glucose can also approach the galactosyl moiety with less restriction. When short-lived E-GA·Glc complexes form upon collision, the reaction is very rapid because the glucose is not restricted by interaction with Trp-999. 1,4-Butanediol is more reactive than glucose (Figures 1 and 2). This is probably because there is less steric hindrance.

The above results indicate that Trp-999 should be important for allolactose production. When lactose is reacted upon by wild type β-galactosidase, the $\beta(1-4)$ bond of lactose is cleaved by the wild-type enzyme and ~50% of the glucose is released (*1*). The C6 hydroxyls of the rest of the glucoses react to form allolactose. However, only W999F-β-galactosidase formed significant amounts of allolactose from lactose (and less allolactose was formed by it than by wild type). Glucose binds very poorly to galactosyl-W999F-β-galactosidase. However, the large k_4 value compensates and the reaction with the short-lived E-GA·Glc complex proceeds so rapidly that some allolactose forms. The other substituted galactosyl-β-galactosidases bind glucose even less well and in those cases even though the k_4 values are large, very little allolactose is formed. This is because the glucose is released so rapidly that the concentration of the short-lived E-GA·Glc complexes is too small for much reaction to take place. Trp-999 of wild type β-galactosidase provides a balance so that some of the glucose cleaved from lactose remains bound long enough for reaction but it positions the glucose so that the high potential rate of reaction resulting from the intrinsic reactivity of the primary C6 hydroxyl is not attained. The combined effect of strong binding, but attenuated transgalactosidic reactivity mediated by Trp-999, ensures that the appropriate amount of allolactose is formed.

Conclusion. Overall, this study shows that Trp-999 is a key residue at the active site of β-galactosidase (Scheme 2). It is important for shallow mode binding to the free enzyme. It affects the degalactosylation rate. It is also important for the transgalactosidic reaction to form allolactose. Substitution essentially abolishes glucose binding to galactosyl-β-galactosidase but the rate at which the transgalactosidic reaction takes place is no longer attenuated.

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BI0270642