His-357 of β-Galactosidase (*Escherichia coli*) Interacts with the C3 Hydroxyl in the Transition State and Helps To Mediate Catalysis

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ABSTRACT: The His at position 357 of β-galactosidase (*Escherichia coli*) was substituted by an Asp, an Asn, a Leu, and a Phe, and studies done with the substituted enzymes showed that the main role of His-357 is to stabilize the transition state by interacting with the C3 hydroxyl. The substituted enzymes were less stable to heat than was wild-type enzyme (40−90% of the activity was lost in 10 min at 52 °C compared to wild-type β-galactosidase which lost no activity), but the gross physical properties of the substituted enzymes at normal temperatures were not changed. There were also no differences in the ability to bind or to be activated by Mg^{2+}. The substitutions (except Asp) did not affect the pK_a for binding substrate in the ground state, but the pK_a of the k_{cat} was altered as would be expected for a residue important for binding the transition state. Substitution by Asp may cause a conformational change at high pH values. Activation energy differences ($\Delta\Delta G_S^{\ddagger}$), as determined by differences in k_{cat}/K_m values, indicated that substitutions for His-357 caused significant destabilizations of the first transition state (for the step in which the galactoside bond is cleaved and the covalent reaction intermediate is formed). This resulted in decreases of up to 900-fold in k_2 for the mononitrophenyl substrates. In contrast, the k_3 values (which depend on the energy level of the second transition state) were not decreased as much (<90-fold). In some cases, the k_3 values even increased (when Asn was substituted for His-357). The importance of His-357 for stabilization of the transition state was confirmed by studies with transition state analogue inhibitors that showed that His-357 forms strong specific interactions with the C3 hydroxyl of the galactose moiety of the transition state. Studies with substrate analogue inhibitors indicated that His-357 is probably not important for the binding of the substrates themselves.

Lac Z β-galactosidase (EC 3.2.1.23) from *Escherichia coli* is a retaining glycosidase that catalyzes the hydrolysis and transgalactosylis of β-D-galactosides (*1*). The reaction probably proceeds via two oxocarbenium ion transition states and a transient covalent galactosyl-enzyme intermediate (*2*). The enzyme is a homotetramer that requires Mg^{2+} or Mn^{2+} and Na^+ or K^+ for maximal activity (*3*−*5*). The amino acid sequences of *lac Z* β-galactosidase and nine other related β-galactosidases (Figure 1) reveal that a residue corresponding to His-357 is universally conserved (*6*−*15*). β-Galactosidase (*E. coli*) also shares homology with the β-glucuronidases, and an equivalent to His-357 is also totally conserved among the β-glucuronidases (*16*−*19*). The three-dimensional structure of β-galactosidase from *E. coli* has been determined to 2.5 Å (*20*). His-357 is located within the active site pocket, and its imidazole side chain forms part of the wall surrounding the active site cavity. The His-357 side chain has a nitrogen atom that is free for interaction and that is oriented into the active site cavity. Preliminary structural studies of β-galactosidase with a transition state analogue inhibitor have shown that the free nitrogen of His-357 is near the hydroxyl of the analogue inhibitor that is equivalent to the C3 hydroxyl of D-galactose (Doug Juers and Brian Matthews, University of Oregon, personal communication).

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	354	355	356	**357**	358	359	360
Clostridium thermosulfurogenes	L	N	R	**H**	Q	S	Y
Rhizobium meliloti	L	N	R	**H**	Q	S	F
Kluyveromyces lactis	V	N	R	**H**	D	H	H
Leuconostoc lactis	V	N	R	**H**	E	W	N
Klebsiella pneumoniae	V	N	R	**H**	E	H	H
Lactobacillus bulgaricus	A	N	R	**H**	E	F	D
Clostridum acetobutylicum	V	N	R	**H**	E	F	S
Streptococcus thermophilus	V	N	R	**H**	E	F	N
***Escherichia coli ebg*[a]**	V	N	R	**H**	D	N	D
Escherichia coli	V	N	R	**H**	E	H	H

a. evolved ß-galactosidase (*15*)

FIGURE 1: Amino acid sequences of β-galactosidases from various organisms that are equivalent to residues 354−360 of the β-galactosidase of *E. coli*.

The β-galactosidase active site has a galactose subsite and an aglycone subsite (*21*). The galactose subsite is specific for compounds that resemble D-galactose (*22*) while the aglycone site of the free enzyme is quite nonspecific (*23*). The C3, C4, and, to a lesser extent, C6 hydroxyl groups of D-galactose are required for tight binding at the galactose subsite. An alteration or absence of any of these hydroxyls causes dramatic binding decreases. Catalysis is entirely lost when the hydroxyls at positions C3 and C4 of D-galactose are changed while alteration at the C6 position causes a greatly decreased catalysis rate. Studies employing deoxy and deoxyfluoro analogues of 2,4-DNPG[1] (*24*) demonstrated that the D-galactose hydroxyl groups at C3, C4, and C6 each contribute at least 16.7 kJ/mol to binding and catalysis.

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We previously reported that His-540 facilitates catalysis in β-galactosidase through interactions with the C6 hydroxyl of D-galactose (*25*) and that those interactions contribute at least 15.4 kJ/mol to transition state stabilization. Our studies on active site conserved His residues have been extended to His-357. We report here the importance of His-357 of β-galactosidase in mediating catalysis through binding interactions with the C3 hydroxyl of the transition state.

EXPERIMENTAL PROCEDURES

Mutagenesis. β-Galactosidases with substitutions for His-357 (except H357L-β-galactosidase) were generated using a modified Kunkel *dut*$^-$ *ung*$^-$ method (*26*). Mutagenesis and cloning strategies were those described by Roth and Huber (*25*). The primers were 5′-dGC GTT AAC CGT **[GA]A[GT]** GAG CAT CAT CC-3′ and 5′-dGC GTT AAC CGT **TTC** GAG CAT CAT CC-3′ (the altered codon is in boldface print, and the two bases in brackets indicate the degeneracy that was introduced to allow for the creation of several site-specific substitutions). H357L-β-Galactosidase was generated using PCR-based site-directed mutagenesis (EXSite PCR-Based Site-Directed Mutagenesis Kit; Stratagene, 1996) of the *lacZ* gene. The procedure followed was that described in the instruction manual supplied by the manufacturer. The mutagenesis primer was 5′-C**T**C GAG CAT CAT CCT CTG CAT GGT-3′.

Enzyme Purification. The β-galactosidases were purified as described by Cupples et al. (*27*) with an additional passage through an FPLC Superose 6 column. Purity was determined by SDS−PAGE (not shown). The enzymes were greater than 98% pure as judged by densitometry scans.

Heat Stability. The enzymes (0.35 mg/mL) were incubated at 52 °C in 50 mM sodium phosphate buffer (with 1 mM $MgSO_4$, 0.04% NaN_3, pH 7.0). Aliquots (60 μL, removed at various times) were diluted into an equal volume of ice-cold TES assay buffer (30 mM TES, 145 mM NaCl, 1 mM $MgSO_4$, pH 7.0) and assayed.

pH Studies. The pH profiles for $-\log K_m$ and $\log k_{cat}$ (ONPG) were determined in buffer (30 mM TES, 50 mM His, 145 mM NaCl, 1 mM $MgSO_4$) at various pH values. The extinction coefficients of oNP were determined from a pH curve.

Enzyme Assays. Activities were followed in TES assay buffer (30 mM TES, 145 mM NaCl, 1 mM $MgSO_4$, pH 7.0) with a Shimadzu UV-2101 PC Spectrophotometer (25 °C). The extinction coefficients used (pH 7.0) were as follows: $\epsilon_{420} = 2.65$ mM^{-1} cm^{-1} for oNP; $\epsilon_{420} = 6.50$ mM^{-1} cm^{-1} for pNP; $\epsilon_{420} = 2.84$ mM^{-1} cm^{-1} for 2,3-DNP; $\epsilon_{400} = 12.60$ mM^{-1} cm^{-1} for 3,4-DNP; $\epsilon_{440} = 3.89$ mM^{-1} cm^{-1} for 2,5-DNP. The dinitrophenol extinction coefficients were determined experimentally. K_m and k_{cat} were obtained using a nonlinear regression program (Enzyme Kinetics, American Chemical Society).

The effect of Mg^{2+} was studied by comparing the rates in the presence of 1 mM $MgSO_4$ to those in the presence of 10 mM EDTA (no added Mg^{2+}) and by equilibrium dialysis. For equilibrium dialysis, wild type and H357F-β-galactosidase were passed (separately) through a Superose 6 column preequilibrated with 30 mM TES buffer (pH 7.0) that was prepared with Milli-Q water and contained 145 mM NaCl and 10 μM $MgSO_4$. Prior to dialysis, the enzymes were concentrated to 2 mg/mL using a Microsep centrifugal concentrator (cutoff 30 kDa). The enzymes were dialyzed against several exchanges of buffer containing 10 μM Mg^{2+} using Spectrapor cellulose dialysis tubing (cutoff 12−14 kDa). After the final buffer change, the enzymes were allowed to dialyze for 24 h to ensure that equilibrium was reached. The final protein concentration was checked to account for any changes that occurred. The enzymes and the final buffer dialysates were analyzed for Mg^{2+} by atomic absorption analysis (Perkin-Elmer 5000).

Steady-State Kinetic Studies. The probable mechanism of the β-galactosidase reaction is shown in Scheme 1. Inhibitors (designated as X) also usually act as acceptors and can react in place of water. Equations 1 and 2 hold at steady state if no inhibitors/acceptors are present (*21*).

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \tag{1}$$

$$K_m = \frac{K_s k_3}{k_2 + k_3} \tag{2}$$

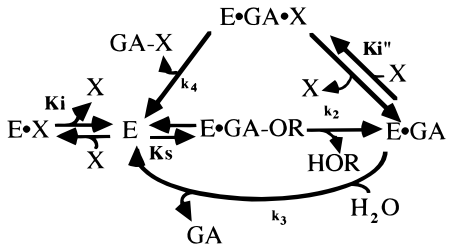
When determining the inhibition constants (K_i), the effect of the inhibitor also acting as an acceptor is taken into account by eq 3 which holds for the disappearance of the total substrate (*21*, *23*).

$$\frac{\text{app}K_m}{\text{app}k_{cat}} = \frac{K_m}{k_{cat}}\left(1 + \frac{[X]}{K_i}\right) \tag{3}$$

K_i values were determined from this equation at several inhibitor (acceptor) concentrations, and the average K_i values are reported.

Studies with acceptors reacting in place of water provide information about the rate-determining steps. If k_2 is rate-

Scheme 1: Postulated β-Galactosidase Mechanism with Inhibitor/Acceptor[a]



[a] The inhibitor/acceptor binds to both the free and the "galactosylated" enzyme. The acceptor can react (k_4) to form adducts. The dots indicate that complexes exist with the enzyme. E, β-galactosidase; GA-OR, substrate; GA, galactose; HOR, aglycone; X, inhibitor/acceptor; GA-X, adduct. The step with the rate constant k_2 is called "galactosylation" while the step with k_3 is called "degalactosylation".

[1] Abbreviations: 2,3-DNP, 2,3-dinitrophenol; 2,5-DNP, 2,5-dinitrophenol; 3,4-DNP, 3,4-dinitrophenol; 2,3-DNPG, 2,3-dinitrophenyl β-D-galactopyranoside; 2,5-DNPG, 2,5-dinitrophenyl β-D-galactopyranoside; 3,4-DNPG, 3,4-dinitrophenyl β-D-galactopyranoside; EDTA, ethylenediaminetetraacetate; FPLC, fast protein liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; oNP, *o*-nitrophenol; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PETG, phenylethyl β-D-thiogalactopyranoside; pNP, *p*-nitrophenol; PNPG, *p*-nitrophenyl β-D-galactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TLC, thin-layer chromatography.

limiting and $k_4 > k_3$, no change in the reaction rate is observed in the presence of the acceptor (one must be careful, however, to ensure that the reason that the rate does not change is not because the acceptor is inert; this was tested by TLC). If k_3 is partially or fully rate-limiting (and $k_4 > k_3$), the apparent k_{cat} will increase as a function of acceptor concentration to a maximum $[k_2k_4/(k_2 + k_4)]$ (*21*). If $k_4 < k_3$, the rate of reaction will slow regardless of the relative values of k_2 or k_3, and no definitive information about whether k_2 or k_3 are rate limiting can be gleaned.

Information about rate-determining steps and of the values of rate constants can also be obtained in other ways. Degalactosylation (k_3) is a common step of reactions of β-galactosidase with galactosyl substrates. Therefore, when k_{cat} values for different substrates are the same, k_3 is probably rate-limiting, and the k_{cat} values are equal to k_3. If the k_{cat} values are significantly different for a series of galactosyl substrates, galactosylation (k_2) is rate-determining for any substrates that have k_{cat} values significantly less than the k_{cat} of the substrate having the highest k_{cat} (because k_3 cannot be any lower than the highest k_{cat} value, by definition). By the same reasoning, if the k_{cat} values differ for different substrates of the same β-galactosidase, the k_3 value is equal to or greater than the largest k_{cat}. Another way of obtaining minimal estimates of k_3 when rate increases resulting from addition of acceptor are too small to detect accurately is to estimate the minimum amount of increase in the apparent k_{cat} that could be accurately detected. In the case of this study we could not accurately detect any rate increase upon addition of 1 M methanol or 1 M propanol. Studies carried out in this laboratory show that we could have accurately detected a 3% increase of the apparent k_{cat} relative to k_{cat}. Thus, methanol or propanol must cause rate increases of less than 3%. Simple mathematical manipulation using this 3% detection threshold indicates that k_3 must be at least 34× as large as the k_{cat} with ONPG in the case of each substituted enzyme.

The k_{cat}/K_m value [equal to k_2/K_s for β-galactosidase with nitrophenyl substrates (*21*, *22*)] is a second-order rate constant for the formation of the first enzyme·transition state complex. Differences in k_{cat}/K_m were used to calculate the $\Delta\Delta G_S^{\ddagger}$ values using eq 4.

$$\Delta\Delta G_S^{\ddagger} = RT \ln \frac{(k_{cat}/K_m)_{mut}}{(k_{cat}/K_m)_{wt}} \qquad (4)$$

The $\Delta\Delta G_S^{\ddagger}$ values indicate how much lower the activation barrier of the first transition state is for wild-type enzyme than for the individual substituted enzymes (*28*−*30*). [The energy required to attain the transition state depends on two factors. First, it depends on the "intrinsic" energy required to achieve the transition state (needed in either the absence or the presence of enzyme). Second, it depends on the ability of the enzyme to bind the transition state and thereby stabilize it. It is assumed that the "intrinsic" energy to achieve the transition state is the same in the substituted enzymes as in the wild-type enzyme, and so the only difference indicated by $\Delta\Delta G_S^{\ddagger}$ is the difference in stability that results from substitution.]

Thin-Layer Chromatography. No detectable increases of the k_{cat} values were found upon addition of inhibitor/acceptor. To show, therefore, that acceptors were actually reacting, the enzyme assays were done in the presence of methanol and propanol and analyzed by TLC. Fifty microliters of enzyme (0.35 mg/mL) was added to 950 μL of 10 mM ONPG and 1 M methanol or 1-propanol in TES buffer (30 mM TES, 145 mM NaCl, 1 mM $MgSO_4$, pH 7.0). After reaction, 2 μL of each reaction mixture was applied to the origin (5 cm K6F Silica Gel 60 Å) and dried. The solvent used for separation was butanol/ethyl acetate/2-propanol/acetic acid/water (1:3:2:1:1) or ethyl acetate/methanol/water (10:2:1). The plates were developed by heat after quickly dipping into a 2% (w/v) orcinol solution in 10% H_2SO_4 (v/v) or into 2% H_2SO_4 (v/v) in methanol. Products were identified by comparison to 10 mM standards of D-galactose, 1-methyl-β-D-galactopyranoside, and ONPG.

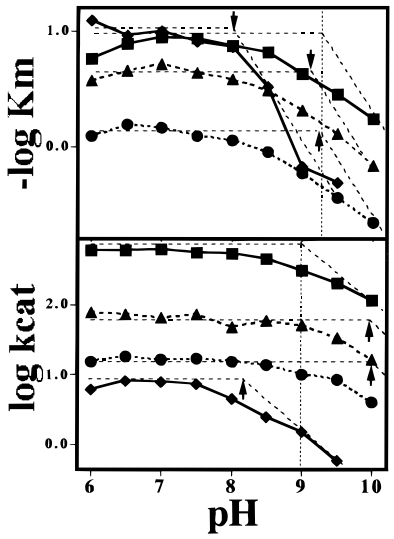


FIGURE 2: Plots of $-\log K_m$ (K_m units = mM) and of $\log k_{cat}$ (k_{cat} units = s^{-1}). (■) Wild-type β-galactosidase; (◆) H357D-β-galactosidase; (●) H357F-β-galactosidase; (▲) H357N-β-galactosidase. The vertical dotted line drawn in each plot represents the pK_a value of the wild-type enzyme. The arrows represent the pK_a values of the substituted enzymes. In each case, the pK_as produced a change in slope of 1.

RESULTS

Heat Stability. The substituted enzymes were somewhat less stable to heat (52 °C) (40−90% of the activity was lost in 10 min at 52 °C, data not shown) than wild-type enzyme (which lost no activity). H357D-β-Galactosidase was the most unstable of the enzymes.

Mg^{2+} Binding and Activation. The k_{cat} of H357F-β-galactosidase (with ONPG) in the presence of Mg^{2+} was 16 s^{-1} while it was 1.1 s^{-1} in the absence of Mg^{2+} (i.e., with 10 mM EDTA). The equivalent values with wild-type β-galactosidase were 620 and 39 s^{-1}. Thus, both enzymes were activated about 16-fold by Mg^{2+}. H357F-β-Galactosidase bound 0.44 Mg^{2+}/monomer (by equilibrium dialysis) whereas the wild-type enzyme bound 0.53 Mg^{2+}/monomer when dialyzed against buffer containing 10 μM Mg^{2+}. Values nearer to 1 Mg^{2+}/monomer would have been expected if the enzyme had been dialyzed against higher concentrations of Mg^{2+}. However, at higher Mg^{2+} concentrations, the Mg^{2+} differences inside and outside of the dialysis tubing are small and thus error-prone.

pH Profiles. Figure 2 shows plots of $\log k_{cat}$ and $-\log K_m$ (ONPG) vs pH. Each profile shown in Figure 2 has one

Table 1: Kinetic and Energetic Values for the Substituted and Wild-Type Enzymes with Several Different Substrates[a]

	H357D	H357N	H357L	H357F	wild
ONPG					
k_{cat} (s^{-1})	7.8	64	5.1	16	620
K_m (mM)	0.13	0.21	0.49	1.25	0.14
k_{cat}/K_m (s^{-1} mM^{-1})	59	290	11	13	4430
$\Delta\Delta G_S$ (kJ/mol)	−10.6	−7.1	−14.8	−14.4	−
PNPG					
k_{cat} (s^{-1})	0.1	0.8	0.4	2.7	90
K_m (mM)	0.003	0.006	0.003	0.083	0.041
k_{cat}/K_m (s^{-1} mM^{-1})	33	130	12	32.5	2200
$\Delta\Delta G_S$ (kJ/mol)	−10.4	−7.1	−12.8	−10.4	−
2,3-DNPG					
k_{cat} (s^{-1})	104	1300	13.1	52	1190
K_m (mM)	0.079	0.20	0.11	0.83	0.15
k_{cat}/K_m (s^{-1} mM^{-1})	1310	6500	120	63	7930
$\Delta\Delta G_S$ (kJ/mol)	−4.4	−0.5	−10.3	−12.0	−
2,5-DNPG					
k_{cat} (s^{-1})	210	1890	10.5	320	130
K_m (mM)	0.23	0.57	0.25	0.49	0.41
k_{cat}/K_m (s^{-1} mM^{-1})	920	3330	42	660	315
$\Delta\Delta G_S$ (kJ/mol)	+2.7	+5.9	−5.0	+1.9	−
3,4-DNPG					
k_{cat} (s^{-1})	4.3	70	4.5	11.4	150
K_m (mM)	0.009	0.014	0.061	0.073	0.16
k_{cat}/K_m (s^{-1} mM^{-1})	480	5010	74	156	915
$\Delta\Delta G_S$ (kJ/mol)	−1.6	+4.2	−6.2	−4.4	−
minimum k_3 (s^{-1})[b]	≥210	≥1890	≥13.1	≥320	≥1190
minimum k_3 (s^{-1})[c]	≥265	≥2180	≥170	≥540	

[a] Also shown (at the bottom) are the minimal estimates of the k_3 values for the various substituted enzymes. [b] The largest k_{cat} value for a substituted enzyme acting on several substrates. [c] From the minimal detectable increase in the apparent k_{cat} (3%).

distinct bend in the alkaline region with a slope change equal to 1. The bends correspond to pK_a values. There are also bends at lower pH values with the −log K_m profiles, but the protein is unstable at low pH values and it is difficult to definitively state that these bends are mechanistically important. The dotted vertical lines on the plots represent the points at which the slopes changed for the wild type and represent the pK_a values of wild type. Arrows indicate the pK_a values of the substituted enzymes. Except for magnitude, the −log K_m profiles with H357F- and H357N-β-galactosidase were essentially like those of wild type (with pK_a values at about pH 9.2). The pK_a value on the −log K_m plot for H357D-β-galactosidase was about 8. In contrast, the log k_{cat} plots showed that the different enzymes had distinctly different pK_a values affecting k_{cat} (pK_a = pH 9 for wild type, 10 for H357F- and H357N-β-galactosidase, and 8.2 for H357D-β-galactosidase).

Kinetic Studies with Nitrophenyl Galactosyl Substrates. The k_{cat}, K_m, k_{cat}/K_m and $\Delta\Delta G_S^{\ddagger}$ values with two mononitrophenyl galactosyl and three dinitrophenyl galactosyl substrates are given in Table 1. The substitutions usually caused decreases in the k_{cat} and negative $\Delta\Delta G_S^{\ddagger}$ values. But there were exceptions in the case of the dinitrophenyl galactosyl substrates. For H357F-, H357N-, and H357D-β-galactosidase with 2,5-DNPG, the k_{cat} values increased, and the $\Delta\Delta G_S^{\ddagger}$ values were positive. With H357N-β-galactosidase and 3,4-DNPG, the $\Delta\Delta G_S^{\ddagger}$ value was positive. With H357N-β-galactosidase and 2,3-DNPG, the k_{cat} increased while the $\Delta\Delta G_S^{\ddagger}$ value was near zero. In general, the k_{cat} values with the mononitrophenyl galactosides (ONPG and PNPG) were decreased more (average of 190×) by the substitutions than the k_{cat} values for the dinitrophenyl galactosides (average of 23×). Also, the $\Delta\Delta G_S^{\ddagger}$ values for H357N-β-galactosidase were less negative than those for the other substituted enzymes in all cases, and the $\Delta\Delta G_S^{\ddagger}$ values for H357D-β-galactosidase were generally intermediate between those for H357N-β-galactosidase and those for H357L- and H357F-β-galactosidase. The k_{cat} values of H357N-β-galactosidase were greater than those for the other substituted enzymes for all substrates except PNPG. Table 1 also presents lower limits of the k_3 values using the reasoning described above.

TLC Analysis. TLC analyses (not shown) indicated that the only detectable products formed by the substituted enzymes in the presence of 1 M methanol or 1-propanol as acceptors with ONPG as the substrate were oNP and the transferolysis products (1-methyl-β-D-galactose or 1-propanyl-β-D-galactose). It is significant that no galactose was detected. The detection limit of D-galactose was such that it would have been detected even if it had only constituted 3% of the total product. The lack of any detectable D-galactose means that transgalactosylis (k_4) was the predominant reaction. Despite this, there was no detectable rate increase. Since the acceptors reacted and the rate did not increase, one must conclude that k_2 is rate-determining.

Inhibition Studies. Table 2 is a compilation of the K_i values for substrate analogue inhibitors. The K_i values for the monosaccharide substrate analogue inhibitors having no aglycone (such as D-galactose, D-glucose, etc.) at the top of Table 2 (with equatorial C3 hydroxyls or with equatorial hydroxyls at the position that the C3 hydroxyl would normally bind) were a little larger on average (4.5×) for the substituted enzymes than for the wild-type enzyme. On the other hand, substrate analogue inhibitors in which aglycones were attached to galactose (IPTG, PETG, lactose) did not show such differences in their K_i values when compared to wild-type enzyme (average is only 1.2× larger). Of particular interest is lactose, the natural substrate of β-galactosidase (its inhibition constant can be determined because it is a slow-acting substrate). The substituted enzymes were inhibited equally well by lactose as was the wild-type enzyme. The bottom section of Table 2 shows that there tended to be only small differences (average is 1.5× larger) between the K_i values of the substituted enzymes and the wild-type enzyme for the competitive inhibitors with non-equatorial C3 hydroxyls or with no hydroxyl at the position that would normally be occupied by the equatorial C3 hydroxyl of D-galactose.

The K_i values of the transition state analogues that have equatorial C3 hydroxyls (top of Table 3) were very much larger (average is >195× larger) for the substituted enzymes than for wild-type enzyme. However, the K_i values for the transition state analogues that have an axial hydroxyl at the position equivalent to the C3 hydroxyl of D-galactose (bottom of Table 3) were not exceptionally different in the case of the substituted enzymes than with wild-type enzyme (average is 1.4× larger; L-arabinolactone inhibition of H357F-β-galactosidase was not included in the average; it was presumed that it binds anomalously due to steric or other reasons).

Table 2: Inhibition Constants (mM) for Substrate Analogue Inhibitors Grouped According to Those That Have an Equatorial C3 Hydroxyl and Those with a Nonequatorial C3 Hydroxyl [in the Most Favorable Binding Modes for These Inhibitors (*22*)]

substrate analogues	H357D	H357N	H357L	H357F	wild
equatorial C3 hydroxyls					
D-galactose	30	65	121	145	10
2-D-deoxygalactose	67	7	152	205	62
2-D-aminogalactose	4	7	3.4	8.1	1.1
D-glucose	>650	280	1040	600	410
D-fucose	385	470	314	550	370
D-talose	18	17	42	40	8
D-threitol	137	85	55	95	7
L-threitol	123	72	3.4	140	33
D-arabitol	51	50	55	220	53
ribitol	48	44	38	110	11
IPTG	0.024	0.02	0.18	0.25	0.08
PETG	0.0003	0.0003	0.0002	0.003	0.0009
lactose	0.16	0.28	1.1	2	1.21
nonequatorial C3 hydroxyls					
L-lyxose	>900	69	–	295	150
L-xylose	250	480	590	720	300
L-arabitol	270	230	190	260	190
glycerol	125	110	107	120	100
xylitol	320	170	240	170	240
L-sorbose	485	1040	410	390	365

Table 3: Inhibition Constants (mM) for Transition State Analog Inhibitors[a]

transition state analogues	H357D	H357N	H357L	H357F	wild
equatorial C3 hydroxyls					
L-ribose[b]	62	13	4.0	7.3	0.12
D-galactonolactone	26	11	>33	14	0.1
nonequatorial C3 hydroxyls					
L-arabonolactone[c]	3.3	41	35	>1000	16
D-xylose[d]	250	480	1510	720	730
L-arabinose[d]	220	360	245	610	240

[a] The inhibitors are grouped according to whether the hydroxyl groups at the C3 position are equatorial or nonequatorial. [b] L-Ribose is a very good inhibitor of wild-type β-galactosidase (*39*). When in the furanose form, L-ribose resembles D-galactose but has a planar "envelope" structure (*40*) resembling what one would expect of the transition state (*39*, *41*) (see Chart 1). [c] When in the 5-membered (∂) ring form, the hydroxyl equivalent to the C3 hydroxyl of D-galactose is axial. [d] When in the furanose form, the hydroxyl equivalent to the C3 hydroxyl of D-galactose is axial.

DISCUSSION

General. His-357 was substituted with Asn, Asp, Leu, and Phe. Analyses of families of homologous proteins show that when His is substituted by another amino acid, it is most often exchanged with an acid or amide (*31*, *32*). Substitution by Asn is more conservative than with Asp because it is polar but neutral—like His in its unprotonated form. In addition, its amido nitrogen is at a similar distance from the main chain as is the ∂-N of His. Asp introduces a negative charge but should still be able to form H-bonds. The side chains of Phe and Leu are roughly similar in size to His but lack hydrogen bond forming capabilities.

The studies reported here show that the main role of His-357 is to stabilize the transition state via interactions with the C3 hydroxyl of the galactosyl moiety of the transition state. It is thought that weak interactions between an enzyme and its substrate become stronger as the geometry changes along the reaction coordinate from the ground state toward the transition state (*34*). The galactosyl moiety in the β-galactosidase reaction is postulated to undergo significant changes in geometry from a chair conformation in the ground state into a near-planar "half chair" conformation in the transition state. The data indicate that His-357 interacts weakly (at best) with the ground state but forms strong interactions with the transition state.

Physical Properties. Substitutions for His-357 did not seriously affect the physical properties of the substituted enzymes. The enzymes precipitated at the same ammonium sulfate concentrations and eluted from ion exchange and gel filtration columns in similar volumes as wild type. In addition, fluorescence spectra (not shown) were identical to wild type. The substituted β-galactosidases were more heat-labile at 52 °C than wild type. A decrease in stability upon active site substitution is not surprising since the integrity of the active site in most enzymes is closely associated with the stability of the enzyme (*33*).

H357F-β-Galactosidase bound Mg^{2+} approximately as well as the wild-type enzyme (*5*) and was activated by Mg^{2+} in a similar fashion as was wild-type β-galactosidase. This showed that the properties of the β-galactosidases with substitutions for His-357 were not due to changes at the Mg^{2+} binding site.

Substitutions with neutral amino acids (Figure 2) for His-357 did not affect the pK_a values associated with the K_m but did affect those associated with the k_{cat}. If it is assumed that the K_m values for the substituted enzymes with ONPG are equivalent to K_s and that the k_{cat} values are equivalent to k_2 (as subsequent discussion will indicate), these results mean that neutral substitutions for His-357 do not affect ground state binding but affect transition state binding and thus k_2. This indicates that the pK_a value of the His-357 interaction with the transition state (which affects k_2) is about 9 (this is at the high end of the range that a pK_a of His could vary) and that the interaction must require that His-357 be protonated (since k_2 decreases as a function of the pK_a). Thus, His-357 could act as a hydrogen donor in an H-bond interaction. Substitutions with Asn or Phe caused the pK_a values associated with k_{cat} to increase to 10. Since neither of these residues has a proton that dissociates, the pK_a of 10 must be due to some other group on the protein. When His-357 was substituted by an Asp, the pK_a value was at pH 8

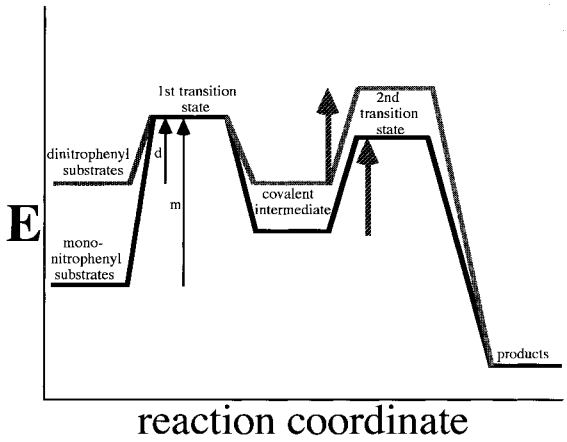
(again rather high for an Asp side chain). If it is assumed that it is the protonated component of Asp that forms an H-bond, the pK_a should decrease. The difference in the case of Asp is that when the pH was higher than the pK_a, the K_m as well as the k_{cat} were affected. Replacement of a His with its positive charge by an Asp (with an opposite charge at high pH) could change the conformation at the active site, and, as a result, the enzyme might no longer bind the substrate. There was some evidence for this. H357D-β-Galactosidase was essentially inactive at pH 10. This could be a result of a critical conformation change.

Contributions of His-357 to Transition State Stabilization. The $\Delta\Delta G_S^{\ddagger}$ values (Table 1) indicate that the activation energy barrier of the first transition state is lower in the case of the wild-type β-galactosidase than for each of the individual substituted enzymes (*28*−*30*) with the mononitrophenyl galactosyl substrates. The stabilization of the first transition state by His-357 with ONPG and PNPG as substrates is about 14−15 kJ/mol and fits into the range expected for residues participating in neutral hydrogen bond pairs (*29*, *35*−*37*). [The values of −7.1 kJ/mol with ONPG and PNPG with H357N-β-galactosidase (Table 1) and of about −10.5 kJ/mol with H357D-β-galactosidase are probably underestimations of the importance of His-357. Some of the interactions with His could be fulfilled reasonably well by Asn (as has also been demonstrated in tyrosyl tRNA synthetase (*38*)) and by Asp.] Simple disruptions in structure caused by substitutions can obviously also alter the stability of the transition state. However, the dramatic effects on transition state analogue inhibition (discussed below), compared to the small effects on substrate and substrate analogue inhibitor binding and the very specific effects on C3 hydroxyl binding in the case of the transition state analogue inhibitors, show that the major $\Delta\Delta G_S^{\ddagger}$ component upon substitution is the loss of the His interaction.

Interactions of β-galactosidase with aglycones were also affected by substitutions for His-357. Both binding and rate were found to differ for the variously substituted enzymes in a manner dependent upon the substrate. For instance, substitution of His-357 by an Asp caused an increase of about 10-fold in binding ($K_m \sim K_s$) of ONPG but a only a 2-fold increase for binding of 2,5-DNPG. Also, the substitution of His-357 by Asn caused a 100-fold decrease in k_2 (k_{cat}) with PNPG but a 15-fold increase with 2,5-DNPG. The k_2 values of different substrates with aglycones of roughly equal leaving ability were affected differently by the different substitutions. In addition, the $\Delta\Delta G_S^{\ddagger}$ values with the dinitrophenyl galactosides were considerably smaller than with the mononitrophenyl galactosides, and TLC studies done at 1 M alcohol concentrations showed that the k_4/k_3 ratio changed as a result of substitutions for His-357. All of these effects are manifestations that show that His-357 of β-galactosidase alters interactions with the aglycone moieties of the substrates. It is possible that substitutions for His-357 actually alter the structure at the aglycone site. However, His-357 is quite far from the aglycone site (*20*), and it is more likely that changes of the interactions at the galactose site upon substitution for His-357 cause the effects. It is known that different nitrophenyl substrates have quite different binding affinities to wild-type β-galactosidase and that this affects the reaction rates differently. For instance, the k_{cat}/K_m of the wild-type β-galactosidase reactions with ONPG is 2× higher, the k_{cat} is 7× higher, and the K_s is 10× higher than with PNPG even though the leaving abilities of *o*-nitrophenol and *p*-nitrophenol are identical. This suggests that different nitrophenol moieties are bound differently in the ground state and that different nitrophenols interact differently in the transition state. This requires that the first transition state includes a quasi-bond with the aglycone. It then follows that if the H-bond interactions with the galactosyl part of the transition state are altered (in position and/or angle) as a result of a substitution, the rates of reaction with various aglycones would be changed. The data, as indicated above, show that this occurs. It can also explain how $\Delta\Delta G_S^{\ddagger}$ values with dinitrophenyl substrates can be smaller than for the mononitrophenyl substrates. The interactions must be such that the transition state is not destabilized as much by substitution for His-357 when the galactosyl part of the transition state is in a quasi-bond with a dinitrophenol as when it is in a quasi-bond with a mononitrophenol. Otherwise, the $\Delta\Delta G_S^{\ddagger}$ values would be affected approximately the same by the substitutions. The acceptor reaction with methanol and propanol also showed that His-357 affects reactions with aglycones since the k_4/k_3 ratio is much greater upon substitution for His-357. Again the effect is likely to be on the binding position and/or angle of the transition state relative to the acceptor position. In this case, however, the effect could also depend on the stability of the covalent intermediate, and this relates to how k_3 is affected. This shall be discussed below.

The Rate-Determining Step. The negative $\Delta\Delta G_S^{\ddagger}$ values found in most cases (especially with ONPG and PNPG) indicate that the substitutions caused destabilizations of the first transition state. The following findings clearly indicated that the losses of activity that were found in most cases (especially with the mononitrophenols) resulted mainly from k_2 decreases with smaller changes of the k_3 values: (1) The rates for each of the substituted β-galactosidases with different substrates were not changed to the same value. The degalactosylation step (k_3) is common to all of the substrates studied, and if that step would have been decreased significantly relative to k_2, the rates for each substrate with each individual substituted enzyme should have been very similar. Since this was not the case, galactosylation (k_2) must have been decreased more than degalactosylation (k_3) by the substitutions for His-357. (2) Methanol and/or 1-propanol did not change the rates of the reactions with the substituted enzymes with ONPG when they were acceptors and adducts with acceptors were the only products (besides nitrophenols) that could be detected (by TLC) in the presence of high concentrations of these acceptors. The reason was that k_2 was rate-limiting. (3) The lowest possible values of k_3 were not nearly as different from the k_3 value of wild-type β-galactosidase (about 1200 s^{-1}) as were the k_2 values. In the case of H357N-β-galactosidase, the lower limit estimate of the k_3 value is actually higher than the value of k_3 for wild-type β-galactosidase. Two methods of estimating the lower limit of k_3 were used. The method of estimation using minimal detectable values of increases in the apparent k_{cat} gave higher k_3 values (especially in the case of H357L-β-galactosidase) than using the highest k_{cat} for a series of substrates (bottom of Table 1). It should be stated that the k_3 values found by both methods of estimation are lower limits and the values of k_3 could be higher than the lower

Scheme 2: Possible Energy Profile for the β-Galactosidase Reaction[a]



[a] Dinitrophenyl substrates are less stable than mononitrophenyl substrates as indicated by the solid arrows (d = arrow representing the activation energy to achieve the first transition state with dinitrophenyl galactosyl substrates; m = arrow representing the activation energy to achieve the first transition state with dinitrophenyl galactosyl substrates). The stabilities of the covalent intermediate and the second transition state are both important in determining k_3. The two arrows of equal height in the area of the second transition state of the profile indicate how an instability of the second transition state created by a substitution for His-357 could be compensated for by a similar instability of the covalent intermediate.

estimates. (4) Dinitrophenyl galactosides in the ground state are quite unstable because dinitrophenols are very good leaving groups (Scheme 2). If k_{cat} is equal to k_2, the k_{cat} values should be larger for the dinitrophenyl substrates because the ground state is unstable. This was the case (Table 1). However, even though the k_2 values are larger, they are not large enough so that k_3 becomes rate-limiting, or the rates for a substituted enzyme with various substrates would have been equal.

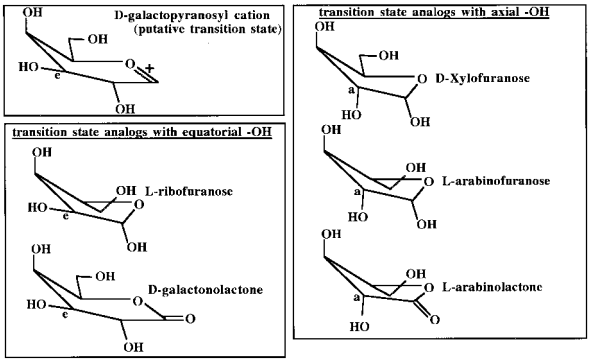
Substitution of a group involved in stabilizing an oxocarbenium ion would actually be expected to have a similar effect on both degalactosylation (k_3) and galactosylation (k_2) because both transition states are thought to have oxocarbenium ion character. However, substitutions for His-357 probably destabilize the covalent intermediate by the same amount as they destabilize the second transition state (Scheme 2) because the covalent intermediate seems to be stabilized in a similar manner by the enzyme as is the transition state (Juers and Matthews, personal communication). If the stability of the covalent intermediate and the stability of the second transition state are decreased by similar amounts, the activation energy to attain the second transition state starting at the covalent intermediate should not be changed (the two arrows in Scheme 2 that are in the second transition state area of the profile are of equal height and show that if both the covalent intermediate and the second transition state are destabilized by an equal amount, the k_3 value would not change). The k_3 could actually be larger after substitution for His-357 (as occurs with H357N-β-galactosidase). If the covalent intermediate is stabilized poorly relative to the stabilization of the second transition state, less energy is needed to attain the second transition state. It is also, of course, possible that His-357 may not interact in the same way with the two different transition states.

It is possible that the substitutions could cause the rate of binding of the substrate to be rate-limiting. However, since the data below clearly indicate that the effect is mainly on interactions with the C3 hydroxyl of the transition state and since binding of substrate in the ground state is not significantly affected, it is unlikely that binding has become rate-determining.

Inhibition by Substrate Analogues. The data indicate that His-357 is only of borderline importance for ground-state substrate binding. If His-357 does interact with the equatorial C3 hydroxyl group of D-galactose, substituted enzymes should bind inhibitors with equatorial C3 hydroxyls less well because the normal interaction is absent. The K_i data for substrate analogue inhibitors with equatorial C3 hydroxyls or equivalent (top of Table 2) show that the inhibitory effect in the ground state for the substrate analogue inhibitors with no aglycones attached (e.g., D-galactose, D-glucose etc.) was decreased somewhat (K_i values increased an average of 4.5×) when substitutions for His-357 were made. On the other hand, the data (bottom section of Table 2) indicate that the interactions normally present between the side chain of His-357 and the equatorial C3 hydroxyl of D-galactose were essentially not affected by the substitutions when the inhibitors did not have equatorial C3 hydroxyls (average increase was only 1.2×). To understand this, it must be realized that L-lyxose, L-xylose, and L-sorbose (bottom section of Table 2) are found almost exclusively in the six-membered pyranose ring form (*39*) and in that form they are like D-galactose or D-talose in structure except that they do not have a C6 hydroxyl group but, more importantly, the C3 hydroxyl is oriented axially. Also, the hydroxyl groups of L-arabitol and xylitol [when these alcohols bind in the energetically most favorable way (*22*)] that are equivalent to the C3 hydroxyl of D-galactose would be oriented in the axial position. In addition, the most favorable mode for glycerol binding to the active site would be such that it would occupy sites at which the hydroxyls of carbons 4−6 of D-galactose would normally bind and thus the glycerol would not have a hydroxyl available to bind to the position that the C3 hydroxyl of D-galactose normally binds.

The substrate analogue inhibitors with aglycones attached (IPTG, PETG, and lactose) inhibited the substituted enzymes about as well as they inhibited the wild-type enzyme (Table 2) even though the C3 hydroxyls were equatorial. In particular, the K_i for lactose was not significantly changed as a result of the substitutions. This trend was also noted in the K_m values (Table 1) for the synthetic substrates which also, of course, are molecules with aglycones attached to D-galactose. If anything, the substitutions caused the K_m values to decrease rather than increase. The presence of an aglycone, therefore, seems to negate the small C3 hydroxyl dependence of His-357 for ground state binding found in the absence of an aglycone. It is possible that the decreases in inhibition seen for the substrate analogues without aglycones may actually be related to transition state binding. When an aglycone is not present, the binding would not be constrained by the aglycone interactions, and this could allow the lone sugar to reorient and form weak interactions similar to those of the transition state. This suggests that His-357 may not actually make any contribution to ground state binding of substrates.

Chart 1: Structures of the Putative Transition State of β-Galactosidase and of Potential Transition State Analogue Inhibitors Used in the Study[a]



[a] e and a refer to equatorial and axial: the configurations of the hydroxyl at the position equivalent to the C3 hydroxyl of D-galactopyranose.

Inhibition by Transition State Analogues. The inhibition brought about by transition state analogues was very different from that caused by substrate analogues and along with the $\Delta\Delta G_S^{\ddagger}$ values discussed above confirmed that His-357 is very important for transition state stabilization. In addition, the transition state analogue inhibition data showed that the stabilization occurs via interaction with the C3 hydroxyl. D-Galactonolactone and L-ribose are good transition state analogue inhibitors (*39*). D-Galactonolactone probably resembles the planar structure of the oxocarbenium ion transition state (Chart 1), and its hydroxyl groups are probably lined up in a similar way as are the hydroxyls of the galactosyl part of the transition state (*40*). L-Ribofuranose (Chart 1) has an envelope structure with carbons 1 and 4 as well as the ring oxygen in the same plane. If bound in the same way as the putative transition state is bound, it would not have a carbon or hydroxyl group at the position normally occupied by the C1 of the transition state (in which carbons 1, 2, and 5 as well as the ring oxygen are in the same plane) and could thus be accommodated by a site that is complementary to a planar transition state. The inhibition by these two transition state analogues with equatorial hydroxyls equivalent to the C3 hydroxyl of D-galactose was decreased an average of $>195\times$ upon substitution for His-357 (top section of Table 3). The differences in K_i values were, however, small (average of only $1.4\times$ larger) for the compounds in the bottom section of Table 3 which are similar in structure to the above transition state analogue inhibitors but have axial hydroxyls at the positions equivalent to that of the C3 hydroxyl. Therefore, the interaction of His-357 with the C3 hydroxyl is very important for transition state stabilization.

CONCLUSION

It is important to establish the roles of individual residues at the active sites of enzymes. The evidence described here strongly suggests that the main role that His-357 of β-galactosidase plays is to stabilize the transition state by interactions with the C3 hydroxyl.

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