The β-Galactosidase (Escherichia coli) Reaction Is Partly Facilitated by Interactions of His-540 with the C6 Hydroxyl of Galactose

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β-Galactosidase with substitutions for His-540 were only poorly reactive with galactosyl substrates. However, the activity with substrates that were like galactose but did not have a C6 hydroxyl group was not decreased much as a result of such substitutions. The loss of transition state stabilization for galactosyl substrates as a result of substitution was between −15.4 and −22.8 kJ/mol but only between +0.34 and −6.5 for substrates that were identical to galactose but lacked the C6 hydroxyl. These findings indicate that an important function of His-540 is to aid in the stabilization of the transition state by forming a stable interaction with the C6 hydroxyl group. This suggestion was strengthened by the results of competitive inhibition studies showing that L-arabinolactone (a transition state analog inhibitor of β-galactosidase without a C6 hydroxymethyl group) was bound as well by the substituted enzymes as by wild type, whereas transition state analog inhibitors that contain C6 hydroxyls (L-ribose and D-galactonolactone) were bound much more poorly by the substituted enzymes than by the wild type enzyme. Substrate analog inhibitor studies showed that His-540 was also important for binding interactions with the C6 hydroxyl group of the ground (substrate) state. The activation by Mg\(^2+\) was the same for the substituted enzymes as for the wild type, and equilibrium dialysis showed that HS40F-β-galactosidase bound Mg\(^2+\) as well as did normal β-galactosidase. The Kd and Ks values seem to have the same pH interactions as wild type enzyme, whereas the k1 interactions are affected differently by pH in the substituted enzymes than in the wild type enzyme. The rate of the “degalactosylation” reaction was affected more by substitutions for His-540 than was the rate of the “galactosylation” reaction. All three substituted β-galactosidases were less stable to heat than was wild type, but HS40N-β-galactosidase was somewhat more stable than the other two substituted enzymes. There were some differences in activity and inhibitory properties that resulted from the different substitutions.

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from Escherichia coli catalyzes hydrolytic and transgalactosylic reactions with β-D-galactosides (Huber et al., 1976). The amino acid (Fowler and Zabin, 1978) and nucleotide (Kalnins et al., 1983) sequences have been determined. The enzyme is a tetramer, and each identical monomer (116,353 Da-1023 amino acid residues) functions independently (Cohn, 1957). Mg\(^2+\) or Mn\(^2+\) and Na\(^+\) or K\(^+\) are required for full catalytic efficiency (Tenu et al., 1972; Case et al., 1973; Huber et al., 1979). The three-dimensional structure of β-galactosidase (Jacobson et al., 1994) shows that the active site is in a deep pocket within a distorted “TIM” barrel. His-540 is located in the wall of the active site cavity, and one of its nitrogens is at the edge of the active site cavity and appears not to be H-bonded to any other group in the free enzyme. A His equivalent to His-540 is conserved (Fig. 1) in every related β-galactosidase that has been sequenced to date (Kalnins et al., 1983; Burchhardt and Bahl, 1991; Buvinger and Riley, 1985; David et al., 1992; Fanning et al., 1994; Hancock et al., 1991; Poch et al., 1992; Schmidt et al., 1989; Schroeder et al., 1991; Stokes et al., 1985). In addition, His-540 is only three residues removed from Glu-537, a residue that probably acts as a nucleophile in the catalysis scheme (Gebler et al., 1992). Recent unpublished results indicate that His-540 is within H bonding distance of the C6 hydroxyl groups of substrate and transition state analog inhibitors.

The active site of β-galactosidase has two subsites. The aglycone subsite lacks specificity, whereas the galactose subsite is very specific (Deschavanne et al., 1978; Huber et al., 1984). Losses in binding at the galactose subsite are very dramatic, and catalysis is completely eliminated if there are changes at positions C3 and/or C4. Changes at C6 also have large effects on binding, but sugars that have hydroxyl groups in the same orientation as α-galactose except for changes at the C6 group are still quite good substrates (Marshall et al., 1977). McCarter et al. (1992) found that the hydroxyl groups of galactose at C3, C4, and C6 each contributed at least 16.7 kJ/mol to binding and catalysis.

Compounds that resemble α-galactose but have a planar shape (e.g. α-D-galactonolactone) or do not have the carbon equivalent to the C1 group that α-galactose has (e.g. α-D-ribose-furanose form) are good inhibitors of β-galactosidase (Lalegerie et al., 1982; Huber and Brockbank, 1987). Their geometries probably resemble the putative planar oxo-carbonium ion transition state. The active sites of enzymes are usually structured to be more complementary to the transition state than to the substrate. Therefore, interactions between an enzyme and its transition state are very important (Fersht, 1985). In this paper we report the importance (for binding and catalysis) of the interactions of His-540 of β-galactosidase from E. coli with the C6 hydroxyl group of both the ground and the transition states.

MATERIALS AND METHODS

Generation of β-Galactosidases with Substitutions for His-540—All the mutagenesis procedures utilized the pBS SK+ variant (Stratagene). The primers used were 5′-GAA TAC GCC (GA)A(AT) GCG ATG GGT A-3′ (the altered codon is shown in bold and the two bases within parentheses indicate the degeneracy that was introduced). All cell cultures were propagated in LB medium at 37°C (LB growth medium consisted of 1% (w/v) Tryptone, 1% (w/v) NaCl, and 0.5% (w/v) yeast extract (pH 7.5 at 25°C)). All media were autoclaved (120°C, 22 psi) for

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20 min before using. If required, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (0.002%, w/v), isoprpyl-thio-β-D-galactopyranoside (0.02%, w/v) ampicillin (50 μg/ml) and/or tetracycline (40 μg/ml) were added to the cooled autoclaved liquid agar before dispensing. The method used for site-directed mutagenesis was a modified version of Kunkel’s de-ung method (Kunkel et al., 1987). A DNA fragment of the lacZ gene containing the codon to be mutated was excised from a plasmid containing the gene (pIP101) using *Cla*I and *ligated* to the phosphorylated primers were annealed to the single-stranded DNA gene, and this was used as the template DNA for mutagenesis. The restriction enzymes methods used were those of Sambrook and co-workers (1989). Single-stranded DNA was isolated using a helper phage (VCS M13, 14297) and was used as the template DNA for mutagenesis.

The phosphorylated primers were annealed to the single-stranded DNA template. T4 DNA polymerase and T4 DNA ligase were added to the phosphorylated primers were annealed to the single-stranded DNA and this was used as the template DNA for mutagenesis. The restriction enzymes methods used were those of Sambrook et al., 1989 except that the enzyme was passed through an FPLC Superose 6 column as a last step. Purification, 2 mM ONPG was used routinely for the assays.

The probable mechanism of β-galactosidase activity includes the formation of [Cat OR] and [Gal OR] complex. The step with *k* 4 is rate-limiting. The rate constant is calculated as large as *k* 4 and, therefore, that *K* 4 (without the acceptor) is essentially equal to *K* 4/(*K* 4 + *K* 5). If the rate increases more than 10-fold it indicates that *k* 4 is at least 10 × as large as *k* 3 and, therefore, that *K* 4 (without the acceptor) is essentially equal to *k* 4. If *k* 4 > *k* 3 and if *k* 4 is partially or fully rate-limiting, the apparent *K* m values will be calculated when calculating the *K* m values. An equation for this calculation is given above.

One can also obtain information about rate-determining steps using inhibitors/acceptors. Alcohol and sugar acceptors can react with both the free and the "galactosylated" enzyme. When bound to the galactosylated enzyme, the inhibitor/acceptor can react (*k* 5) to form galactosyl adducts. The dote indicate that some sort of complex exists with the enzyme. The inhibitory action must be considered when calculating the *K* m values. The inhibitor/acceptor present is shown on Scheme 1. Molecules that act as competi-

Abbreviations used: TES, Tris(hydroxymethyl)aminomethane; 2-aminoethanesulfonic acid; ONPG, o-nitrophenyl-β-D-galactopyranoside; PNPG, p-nitrophenyl-β-D-galactopyranoside; PNPA, p-nitrophenol-α-L-arabinopyranoside; PNPF, p-nitrophenol-β-D-fucopyranoside; ONP, o-nitrophenyl-β-D-galactoside; PETG, phenyl ethylthio-β-D-galactopyranoside.

2 Deaminases used: TES, Tris(hydroxymethyl)aminomethane; 2-aminoethanesulfonic acid; ONPG, o-nitrophenyl-β-D-galactopyranoside; PNPG, p-nitrophenyl-β-D-galactopyranoside; PNPA, p-nitrophenol-α-L-arabinopyranoside; PNPF, p-nitrophenol-β-D-fucopyranoside; ONP, o-nitrophenyl-β-D-galactoside; PETG, phenyl ethylthio-β-D-galactopyranoside.
k_{s2} the rate of the reaction will slow down regardless whether k_{1} or k_{2} is slower.

The energy needed to attain the transition state can be estimated (Fersht, 1974). The k_{cat}/K_m value (equal to k_{cat}/K_m with β-galactosidase) is a second order rate constant for the formation of the enzyme-transition state complex (starting from free enzyme and substrate) and thus RTln(k_{cat}/K_m) is the energy needed to attain the transition state. The equation below (Fersht and Leatherbarrow, 1987), therefore, gives the difference of the energetic contribution of a substituted side chain “R” to the formation of the transition state when compared to the wild type enzyme. The equation assumes that the activation energy required for bond breaking/making does not change significantly in the substituted enzyme as compared with the wild type enzyme.

$$\Delta G^{\pm} = RT \ln \left(\frac{k_{cat}K_m}{k_{cat}K_m}\right)$$

(Eq. 2)

Thus, the differences in the k_{cat}/K_m values and the subsequent differences in the ΔG^{\pm} values are very important indications of the contribution of a residue to catalysis. This theory applies for the formation of the first transition state. If there is an additional transition state and if more energy is required to attain that transition state, the net effect will be to slow down the rate. If the additional transition state needs less energy to form, the overall rate will not slow down (but it will of course also not be any faster).

Equilibrium Dialysis—Enzyme solutions were passed through a Superose 6 column pre-equilibrated with buffer (pH 7.0) made up in Milli-Q water and containing 30 mM TES, 145 mM NaCl, and 10 mM MgSO_4. The enzymes were concentrated to 2 mg/ml using a Microsep centrifugal concentrator (cut-off 30 kDa), and the solution was placed into Spectra/Por cellulose dialysis tubing (cut-off 12–14 kDa). The enzymes were then dialyzed extensively against the buffer containing 10 mM MgSO_4. After the final buffer change, the enzyme was dialyzed for a 24-h period to ensure that an equilibrium was reached. The final concentration of the protein was again checked to account for changes that may have occurred during dialysis. The enzyme and final buffer dialysates were analyzed by atomic absorption to determine the amount of Mg^{2+}.

pH Studies—The k_{cat}, K_m, and k_{cat}/K_m values of the substituted β-galactosidases were determined at various pH values. Fixed time assays were used for all the pH profile analyses. The reactions were stopped with 2 volumes of 1 M Na_{2}CO_3. This increased the pH to 11 and stopped the reaction. Using this method, all the rates could be determined using a single extinction coefficient since the final measurements were done at the same pH. The extinction coefficients used for these assays were determined with ONP standards.

Heat Stabilities—The enzymes (substituted and wild type) were placed (0.35 mg/ml) into a 50 mM sodium phosphate buffer (pH 7.0, 1 mM MgSO_4) and incubated at 52°C. At various times, 6-μl samples were removed and diluted into an equal volume of ice-cold TES buffer (30 mM TES, 145 mM NaCl, 1 mM MgSO_4, pH 7.0). These enzymes were further diluted, if necessary, immediately before assay.

RESULTS

Assays of Substrates with Changes at C6—Table I gives the k_{cat}, K_m, k_{cat}/K_m and ΔG^{\pm} values for the substituted enzymes and the wild type enzyme with ONPG, PNPG, PNPA, and PNPF. Note that the ΔG^{\pm} values with ONPG and PNPG are significantly larger than those with PNPA and PNPF.

Inhibition by Transition State Analogues—Transition state analog inhibitors with C6 hydroxyl groups inhibited the substituted enzymes much more poorly than they inhibited wild type enzyme (Table II) (except for H540N-β-galactosidase interactions with l-ribose). On the other hand, there was not much difference between the inhibition of the wild type and the substituted enzymes by l-arabinolactone, which is similar to o-galactonolactone but does not have a C6 hydroxyl methyl group.

Inhibition and Acceptor Studies—Table III shows the K_i values for competitive inhibition by various sugars and sugar derivatives. In general, the values of inhibition by the compounds were affected much more by the substitutions if a C6 hydroxyl was present than if it was not present. The differences were usually greater when the His was replaced by Phe than when replaced by Asn or Gli. Some of the inhibitors also acted as acceptors and increased the rates of reaction (Table IV).

ensure that these rate increases were due to transference (acceptor reaction) or reaction in the presence of 1 M methanol. In the case of every substituted β-galactosidase, a compound that migrated as did authentic methylgalactopyranoside was produced in the presence of the methanol.

pH Profiles—Fig. 3 shows that except for magnitudes the dependence of k_{cat}/K_m on pH for two of the substituted enzymes was not much different from wild type. There were, however, definite differences in the K_m and k_{cat} profiles.

Mg^{2+} Activation and Binding—The kinetic results are shown on Table V. The removal of Mg^{2+} had similar effects on the magnitude of the k_{cat} and K_m values in the substituted enzymes as it did in the wild type enzyme. In addition, H540F-β-galactosidase bound 0.49 Mg^{2+} per monomer, whereas wild type β-galactosidase bound 0.53 Mg^{2+} per monomer when di-
alyzed in 10 μM Mg²⁺. The values would probably have been closer to 1 Mg²⁺ per monomer if higher Mg²⁺ concentrations had been used, but at higher concentrations of Mg²⁺, the differences of Mg²⁺ concentrations inside and outside the dialysis tubing would have been small, and it would have been hard to measure the differences.

Heat Stabilities—Fig. 4 shows that all three of the substituted β-galactosidases were more stable than wild type β-galactosidase. H540N-β-galactosidase was, however, a little more stable than Glu which is the other two substituted enzymes.

DISCUSSION

The lacZ genes from E. coli and from some other related β-galactosidases have been sequenced (Kalnins et al., 1983; Burchardt and Bahl, 1991; Buvinger and Riley, 1985; David et al., 1992; Fanning et al., 1994; Hancock et al., 1991; Poch et al., 1992; Schmidt et al., 1989; Schroeder et al., 1991; Stokes et al., 1985), and they share significant homology. These β-galactosidases also share extensive homology with prokaryotic (E. coli) and eukaryotic (rat, mouse, and human) β-glucuronidases (Galagher et al., 1988; Jeffson et al., 1986; Oshima et al., 1987; Nishimura et al., 1986; see Poch et al., 1992 for alignment with β-galactosidases). Several His are conserved among the various sequences, and one of the conserved His is at position 540 (Fig. 1). Histidines are often important at the active sites of enzymes since they can readily form H bonds, they have the capacity to act as acid/base catalysts at pH values that are optimal for enzyme action, they are good nucleophiles, and they can function as ligands for metal binding. Their H bonding capacity means that they could be important for substrate binding and transition state stabilization. The His-540 of β-galactosidase (E. coli) was substituted by Phe, Asn, and Glu in these studies. The Phe was introduced to eliminate the possibility of forming H bonds and because Phe and His are approximately of the same size and His does have some aromatic properties. The Asn was introduced because an amido group can form H bonds similar to those of His and because the distance between the α-carbon of His and the N of His is similar to the distance between the α-carbon of Asn and the amido nitrogen of Asn (Fig. 5). Glu was introduced because we thought that it could interact somewhat like His since it can form H bonds, it can act as a general acid/base catalyst, it can act as a nucleophile, and it can function as a ligand for metal binding. It does, however, introduce a negative charge, and it has a different geometry than does His. The three-dimensional structure (Jacobson et al., 1994) clearly shows that His-540 is at the active site and that one of its nitrogens is available for hydrogen bonding.

Substitutions for His-540 caused the kcat values of the reactions with galactosyl substrates to decrease by about 1000-fold or more (Table I). For substrates that were identical to galactose but did not have C6 hydroxyl groups, the losses of the kcat values were much smaller. In most cases the Km values of the substituted enzymes were relatively small. Since Km = (k2Kf)/(k2 + k3) in the case of β-galactosidase, small Km values either mean that binding of substrate is good or that k3 is small relative to k2. The kcat/Km values were decreased in a similar fashion to the way that the kcat values were, but the differences with the substrates that did not have C6 hydroxyl groups were very small (15-fold at the most). The data shown on Table I indicate that the ΔΔG‡ values that resulted from substitutions for His-540 were about 10–20 kJ/mol larger when a C6 hydroxyl was present than when the hydroxyl was absent. ONPG and PNPG have intact C6 hydroxyl methyl groups, whereas PNPA is similar to β-galactosidase but does not have a C6 hydroxyl methyl group. PNPF is similar to β-galactosidase but it does not have a C6 hydroxyl group. The differences in ΔΔG‡ values with the two classes of substrate strongly suggest that His-540 is involved in binding to the C6 hydroxyl of the transition state. The salient point is that the deleterious effects of the substitutions for His-540 on the kcat/Km and ΔΔG‡ values were much smaller when the C6 hydroxyl group was absent. The differences in ΔΔG‡ values between substrates with and without C6 hydroxyl groups were greatest with H540F-β-galactosidase. This is expected since Phe can't form hydrogen bonds whereas Asn and Glu may be able to participate in H bonds.

In every case (except for the inhibition of H540N-β-galactosidase by l-ribose) substitutions for His-540 led to large losses of the binding ability in the cases of β-galactonolactone and l-ribose but not in the case of l-arabinolactone (Table II). l-Arabinolactone does not have a C6 hydroxyl methyl group while the other two compounds do. These compounds are considered to be transition state analog inhibitors (Huber and Brockbank, 1987). This is further evidence that His-540 stabilizes the transition state via interactions with the C6 hydroxyl of galactose. The relatively good inhibition of H540N-β-galactosidase by l-ribose may be due to favorable H bond interactions of some type that are allowed with the amido nitrogen of Asn.

![Fig. 3](http://www.jbc.org/) pH profiles. A, the kcat values found at various pH values. B, the Kcat (mM) values found at various pH values. C, the kcat/Km values found at various pH values. The data for the kcat and the kcat/Km values were normalized, and the maximal value for each enzyme was equated to 100%. ■ wild type; ● H540F-β-galactosidase; ▲ H540N-β-galactosidase.
Importance of His-540 for the β-Galactosidase Reaction

| TABLE V | The effects of Mg²⁺ on the Kₘ (mM) and kₗₐₜ (s⁻¹) values of the substituted β-galactosidases and on wild type β-galactosidase |
|---------|-------------------------------------------------------------------------------------------------------------------------------------|
|         | H540F | H540N | H540E | Wild type |
| 10 mM EDTA | kₗₐₜ | Kₘ | kₗₐₜ | Kₘ | kₗₐₜ | Kₘ | kₗₐₜ | Kₘ | kₗₐₜ | Kₘ | kₗₐₜ | Kₘ |
| 0.02 | 1.91 | 0.03 | 0.6 | 0.04 | 0.67 | 39 | 0.57 |
| 0.1 | 0.11 | 0.7 | 0.08 | 0.66 | 0.14 | 620 | 0.12 |

There was also a differential effect on inhibition by substrate analog inhibitors (Table III) depending upon whether a C6 hydroxyl was present (except again for the inhibition of H540N-β-galactosidase by some of the compounds). These data show that His-540 also interacts with the C6 hydroxyl of the substrate in the ground state.

In every case, when Mg²⁺ was removed, losses in activity were similar to the effects on wild type β-galactosidase (Table IV). In addition, equilibrium dialysis showed that H540F-β-galactosidase bound as much Mg²⁺ as did wild type β-galactosidase in the presence of 10 µM Mg²⁺. Thus His-540 is not involved in Mg²⁺ binding.

The substituted enzymes had different pH profiles for Kₘ and kₗₐₜ whereas the Kₘ/kₘ profiles for the substituted enzymes were very similar to those of wild type (except for magnitude). The Kₘ value (k₂/Kₘₐₜ(k₂ + k₃)) and the kₗₐₜ value (k₂/Kₘₐₜ(k₂ + k₃)) contain k₂ whereas the kₘ value in Kₘ/kₘ is canceled out (Kₘ/kₘ = Kₘ/kₘ). Thus, these data suggest that the “degalactosylation” (k₃) reaction is affected differently by pH in the substituted enzymes than in wild type enzyme whereas the way k₂ and k₅ interact as the pH is changed is not affected by the substitutions.

Some of the “inhibitors/acceptors” significantly speeded up the rates of reaction with ONPG (Table IV; see also Scheme I). In particular glycerol and glucose were good activators. Since the rate increases, kₗₐₜ must be at least partially rate-limiting. An analysis was done on H540E-β-galactosidase to find the rate extrapolated to an infinite concentration of glucose. The extrapolated rate is equal to k₅K₅/k₅(1 + k₃) (Deschavanne et al., 1978), and the value of this for H540E-β-galactosidase was 15 s⁻¹. This means that k₅ is equal to or greater than 15 s⁻¹ for this enzyme. Since the kₗₐₜ without glucose for H540E-β-galacto-

- **Fig. 4.** The heat stabilities as functions of time. The enzymes (0.35 mg/ml) were placed into a 50 mM sodium phosphate buffer (pH 7.0, 1 mM MgSO₄) and incubated at 52°C. Samples (60 µl) were removed and diluted into an equal volume of ice-cold TES buffer (30 mM TES, 145 mM NaCl, 1 mM MgSO₄, pH 7.0). The enzymes were further diluted, if necessary, immediately before assaying. ■●■, wild type; ▲▼▲, H540F-β-galactosidase; ●▼●, H540N-β-galactosidase; ●●●, H540E-β-galactosidase.

- **Fig. 5.** The structures of His and Asn showing that the electron pairs of the ¹N of His and the amido N of Asn are approximately equi-distant from their respective α-carbons.
hydroxyl of the substrate and the transition state of β-galactosidase. The data show that this is important both for binding the substrates and for stabilizing the transition state of the reaction. The magnitude of the effects observed shows that these interactions are obviously a significant component of the β-galactosidase reaction. The data also showed that the stabilizing effect of His-540 on the C6 group of the transition state may be more significant for the degalactosylation step than for the galactosylation step. Recent structural work† shows that the His-540 side chain is indeed within H bonding distance of the C6 hydroxyl of galactosyl inhibitors.

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