Determination of the Roles of Glu-461 in β-Galactosidase (Escherichia coli) Using Site-specific Mutagenesis*

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Site-directed substitutions (Asp, Gly, Gln, His, and Lys) were made for Glu-461 of β-galactosidase (Escherichia coli). All substitutions resulted in loss of most activity. Substrates and a substrate analog inhibitor were bound better by the Asp-substituted enzyme than by the normal enzyme, about the same for enzyme substituted with Gly, but only poorly when Gln, His, or Lys was substituted. This shows that Glu-461 is involved in substrate binding. Binding of the positively charged transition state analog 2-aminoalactose was very much reduced with Gly, Gln, His, and Lys, whereas the Asp-substituted enzyme bound this inhibitor even better than did the wild-type enzyme. Since Asp, like Glu, is negatively charged, this strongly supports the proposal that one role of Glu-461 is to electrostatically interact with a positively charged galactosyl transition state intermediate. The substitutions also affected the ability of the enzyme to bind L-ribose, a planar analog of n-galactose that strongly inhibits β-galactosidase activity. This indicates that the binding of a planar "galactose-like" compound is somehow mediated through Glu-461. The data indicated that the presence of Glu-461 is highly important for the acid catalytic component of k₃ (glycosyl bond cleavage or "galactosylation"), and therefore Glu-461 must be involved in a concerted acid catalytic reaction, presumably by stabilizing a developing carbonium ion. The k₃ values with o- and p-nitrophenyl-β-D-galactopyranoside as substrates varied more or less as did the k₄ values, indicating that most of the glycolytic bond breaking activity found for the enzymes from the mutants with these substrates was probably a result of strain or other such effects. The k₃ values (hydrolysis or "degalactosylation") of the substituted enzymes were also low, indicating that Glu-461 is important for that part of the catalysis. The enzyme with His substituted for Glu-461 had the highest k₄ value. This is probably a result of the formation of a covalent bond between His and the galactosyl part of the substrate.

β-Galactosidase from Escherichia coli is a disaccharidase which catalyzes the hydrolysis and transgalactosylases (Huber et al., 1976) of β-D-galactopyranosides. Its mechanism of action has not been firmly established, but it has been suggested that it functions in a somewhat analogous way to lysozyme (Sinnott, 1978). Thus, it has been proposed that β-galactosidase has a group which acts as a general acid (donating a proton to the glycosidic oxygen) and a group which stabilizes a galactosyl intermediate, allowing H₂O to react. Studies have shown that the group which acts as the general acid is probably Tyr-503 (Ring et al., 1986, 1988). Wallenfels and Malhotra (1961) suggested that there is an imidazole group at the active site of β-galactosidase that covalently stabilizes a galactosyl intermediate for reaction with water, but other researchers (Tenu et al., 1971; Sinnott and Withers, 1974; Sinnott and Souchard, 1973; Sinnott, 1978) proposed that a carboxyl group acts as a counter ion to a transition state carbonium ion form of galactose. The presence of a negative counter ion was further supported by studies (Huber and Gaunt, 1982; Legler and Herrchen, 1983) which showed that amino sugars and amino alcohols are very good competitive inhibitors of β-galactosidase (especially if the structures of the sugars or alcohols resemble D-galactose). In 1984, Herrchen and Legler used an irreversible active site-directed inhibitor (conduritol-C-cis-epoxide) to identify Glu-461 as a residue with a carboxyl group that might be involved at the active site of β-galactosidase.

In addition to stabilizing a transition state carbonium ion, studies of Sinnott and Souchard (1973), Rosenberg and Kirsch (1981), and Withers et al. (1988) have provided some very good evidence that the putative active site residue with the negative charge also forms a transient covalent bond with the transition state carbonium ion form of galactose. Sinnott and Souchard (1973) proposed that this is necessary to prevent back reaction with aglycon products which are not rapidly released from the enzyme.

Bader et al. (1988) showed that site-specific substitution of Glu-461 with Glu resulted in the loss of >99% of the β-galactosidase activity in a purified preparation. Cupples and Miller (1989) introduced 12 additional substitutions for Glu-461 and showed that all of them decreased the activity of β-galactosidase (by at least 90%) in crude preparations. We describe here the purification and kinetic analysis of five of these β-galactosidases. The purpose of this study was to clarify the role of Glu-461 in β-galactosidase. This study shows that Glu-461 is important for essentially every part of the β-galactosidase mechanism.

**MATERIALS AND METHODS**

Chemicals—Lactose, ONPG, 1 PNPG, TES, Tris, hexamethyldisilazane, trimethylchlorosilane, EDTA, phenylmethylsulfonfyl fluoride.

1 The abbreviations used are: ONPG, o-nitrophenyl-β-D-galacto-
histidine, IPTG, L-ribose, and 2-amino-D-galactose were purchased from Sigma. Other chemicals were purchased from Fisher or similar sources. The purest reagents possible were always obtained.

**Enzyme Production and Purification—β-Galactosidases with amino acid substitutions for Glu-461 were produced as described previously (Bader et al., 1988; Cupples and Miller, 1988) using site-specific mutagenesis to alter the appropriate nucleotides in the E. coli lacZ gene.**

Cells were grown overnight (37 °C with 200-rpm agitation) in Fernbach flasks containing double yeast/Tryptone medium and were harvested by centrifugation at 2,000 × g for 5 min. These cells were then suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.04% NaN₃, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) broken by two passages through a French press (0–4 °C). The suspension was diluted to a protein concentration of 70 mg/ml, and streptomycin sulfate was added (5%, w/v). This was stirred slowly at 4 °C for 2 h, and the mixture was centrifuged at 16,000 × g for 30 min to remove the nucleic acids. The supernatant was diluted to a protein concentration of 18 mg/ml. Solid ammonium sulfate was added to bring the solution to 25% saturation (the pH was maintained between 7.0 and 7.2 by the addition of NH₄OH).

After being slowly stirred for 30 min, the suspension was centrifuged at 16,000 × g for 30 min. The supernatant was then brought to 43% saturation with ammonium sulfate and again stirred and centrifuged. The pellet of this 43% saturation step was dissolved in 80 mM Tris (pH 7.5) with 1 mM MgCl₂, 1 mM 2-mercaptoethanol, and 0.1 mM EDTA. This was dialyzed against large volumes of the same buffer (three times) and then applied to a 2.5 × 40-cm DEAE Bio-Gel A column. The column was washed with 1 liter of the Tris buffer, and then a 1.2-liter NaCl gradient (0.05–0.2 M NaCl) was used to elute the protein. The fractions containing activity (all of the enzymes had some ONPG activity) were concentrated by bringing the solution to 50% saturation with (NH₄)₂SO₄. The resuspended pellet was applied to a fast protein liquid chromatography Superose-12H™ column (Pharmacia LKB Biotechnology Inc.) and eluted with the TES assay buffer (no substrate) with 0.04% sodium azide and 1 mM mercaptoethanol (for storage). Tubes containing activity were analyzed by SDS-PAGE (8–25% acrylamide), and tubes that contained >95% pure monomer were pooled and used for analysis.

**Kinetic Mechanism—** Diagram 1 shows a proposed mechanism of action of the enzyme in the presence of a nucleophilic acceptor. The rate constant k₄ is for “galactosylation” (the breakage of the glycosidic bond). It is thought that Tyr-503 acts as a general acid catalyst in this step (Ring et al., 1985, 1988). Since Glu-461 is probably at the active site (Herrchen and Legler, 1984; Bader et al., 1988; Cupples and Miller, 1988), it could be important for stabilization of a positively charged carbonium ion form of galactose. In addition, Sinnott and Souchard (1973) and Rosenberg and Kirsch (1961) have suggested that the carbonium ion form of galactose probably collapses to form a transitory covalent bond with the carboxyl stabilizing group.

For this mechanism (in the absence of nucleophile) with either ONPG or PNPG as substrate, the following equations hold; kₑ₄ = k₃k₄/(k₃ + k₄) and Kₑ = K₄/(k₃/k₄ + k₄). It follows that when k₃ is rate-determining, the values become kₑ₄ = k₃ and Kₑ = K₄, and when k₄ is rate-determining, the values become kₑ₄ = k₄ and Kₑ approaches 0.

The effect of nucleophile (which replace water) on the rate of reaction can be used to determine whether k₃ is rate-determining (Deschavanne et al., 1978; Huber et al., 1984). This can be seen from inspection of the following equation which predicts how the apparent kₑ₄ value changes as a function of the added nucleophile [N]: apparent kₑ₄ = k₄/(k₃/k₄ + k₄(N)/Kₑ(N))/k₄ + k₃/k₄(N) + k₄(k₄/N)/Kₑ(N)). If k₃ and k₄ are both large relative to kₑ₄, the rate of the reaction will increase as a function of the added nucleophilic concentration. If, on the other hand, k₄ is small relative to kₑ₄, the rate of the reaction will not increase even if k₄ is large. We found that azide ion was a very diagnostic nucleophile for the purpose of finding whether k₃ or k₄ is rate-determining. The kinetics showed that k₄ for azide ion was large; and gas chromato-
Two-hundred μl of this layer was added to 1 ml of toluene, and any precipitate that formed was removed by centrifugation at maximum speed in an Eppendorf microcentrifuge for 30 min. The supernatant was transferred to vials for analysis, and the silylated sugars were separated with a Varian 6900 chromatograph equipped with an automatic sampler. A J & W Scientific fused capillary DB-1 column (50 m) with a 0.322-mm inner diameter and a 0.25-μm film was used. A flow rate of 1 ml/min helium was used with a temperature gradient, and detection was by a flame ionization detector. Retention times of peaks were compared to those of known sugars, and the amounts precipitate that formed was removed by centrifugation at maximum speed in an Eppendorf microcentrifuge for 30 min. The supernatant was transferred to vials for analysis, and the silylated sugars were quantitated in comparison to known standard concentrations. Units are defined as micromoles of product/minute.

pH Effects—Values of $k_{\text{cat}}$ and $K_m$ were determined over the pH range of 5.5 to 10.0 in the TES assay solution buffered at 25 °C. Despite the lack of good buffering capacity at some pH values, the pH values were stable.

Divalent Metal Effects—Studies to determine the effect of the absence of bivalent metal were carried out in the TES assay buffer without Mg" but with 10 mM EDTA present. The enzymes were first added to the buffer without substrate for 30–60 min so that the bivalent metals would have a chance to be totally equilibrated by the EDTA (the removal of Mg" is slow (Tenu et al., 1972)), and the reaction was then started by adding substrate.

Stability Studies—The enzyme (0.5 mg/ml) was incubated in the TES assay buffer in a 55 °C water bath. Aliquots (50 μl) were removed at intervals and placed into tubes which were in ice. After assaying, the percent activity remaining was determined as a function of time.

RESULTS

Purification—Fig. 1 is an SDS-PAGE analysis of aliquots from each of the enzymes. The enzymes were highly pure and migrated to the same position as wild-type β-galactosidase. The β-galactosidases were found to precipitate in the same ammonium sulfate cuts in which the wild-type enzyme precipitated, and they eluted from columns at similar positions as did the wild-type enzyme.

Stability—Fig. 2 shows enzyme stability at 55 °C. The enzymes from the mutants were less stable than the wild-type β-galactosidase. All of the enzymes from the mutants were entirely stable when kept in assay medium for 1 day at 25 °C.

Kinetic Constant Values—Table I shows kinetic values obtained for the purified β-galactosidases using ONPG and PNPG. The enzymes from mutant sources had much lower activities than did wild-type β-galactosidase. E461H-β-Galactosidase activity (as expressed by $k_{\text{cat}}$) was relatively high (~6% of normal with ONPG and 2% of normal with PNPG), whereas E461D-β-galactosidase activity was very low (0.013% of normal with ONPG and 0.008% of normal with PNPG).

Except for the $K_m$ of E461H-β-galactosidase (which was a little larger than that of the wild-type enzyme), the $K_m$ values with ONPG were all much lower than the $K_m$ of the normal enzyme. The $K_m$ value for E461Q-β-galactosidase was particularly low. When PNPG was substrate, the $K_m$ values were also lower (except again for E461H-β-galactosidase), but with this substrate, they were not decreased by such a large factor. Since $K_m = k_2/(k_1 + k_3)$, the low $K_m$ values were probably due to the fact that the $k_3$ values were much lower than the $k_2$ values rather than because $K_m$ was small. Because only lower limits of $K_m$ were found in some cases, only lower limits of $K_m$ were also obtained.

With ONPG as substrate, lower limits of $k_2$ were obtained in every case from the maximum rate that was observed upon addition of a high concentration of azide. The lower limit for E461Q-β-galactosidase shown is probably a substantial underestimate of the actual value obtained since the $K_m$ was very low in that case. With PNPG as substrate, $k_2$ was rate-limiting for E461D-, E461G-, and E461H-β-galactosidases. Therefore, for these enzymes, actual $k_2$ values with PNPG could be obtained.

Only a lower limit of $k_2$ for E461Q-β-galactosidase could be established. In the case of E461K-β-galactosidase, the PNPG $k_{\text{cat}}$ in the presence of 10 mM azide was essentially one-tenth of the $k_{\text{cat}}$ value with ONPG as substrate when 100 mM azide was present. This indicates that $k_3$ is at least 10 times as large as the PNPG $k_{\text{cat}}$ found with 10 mM azide, and the $k_{\text{cat}}$ (being 10 times lower) would therefore essentially be equal to $k_3$.

Values of $k_{\text{cat}}$ were assumed to be equal to $k_3$ if adding azide increased the $k_{\text{cat}}$ more than 10-fold because this meant that the value of $k_2$ was at least 10 times as large as that of $k_3$. This was the case for E461G-, E461Q-, and E461K-β-galactosidase with ONPG as substrate. When PNPG was the substrate, this was the case for E461Q- and E461K-β-galactosidases. As expected, the ONPG $k_{\text{cat}}$ values for these latter two enzymes were essentially equal to the PNPG $k_{\text{cat}}$ values (because $k_{\text{cat}} = k_3$ in each case). For E461D- and E461H-β-galactosidases, we were able to calculate ranges of values for $k_{\text{cat}}$. The gel had a 8-25% acrylamide gradient. The lanes w, D, G, Q, H, and K, E461D-, E461G-, E461Q-, E461H-, and E461K-β-galactosidases, respectively.
two enzymes, and the equation for $E_{461H-\beta\text{-galactosidase}}$ were determined using the fact that $E_{461D-\beta\text{-galactosidase}}$ and $42 \text{s}^{-1}$ for $E_{461H-\beta\text{-galactosidase}}$.

than $0.16 \text{s}^{-1}$ and greater than $71 \text{s}^{-1}$ for $E_{461H-\beta\text{-galactosidase}}$ using the fact that the $k_2$ for $E_{461D-\beta\text{-galactosidase}}$ is greater than $0.10$ and $0.27 \text{s}^{-1}$, whereas that for $E_{461H-\beta\text{-galactosidase}}$ is $0.10-0.27 \text{s}^{-1}$. A good overall indication of the values between $42$ and $103 \text{s}^{-1}$. A good overall indication of the values obtained in those cases. If this was not the case, the $K_a$ values were calculated or true values of $K_m$ were small compared to normal magnitude smaller than the $K_m$ value of the wild-type enzyme. In general, the $K_a$ values for IPTG and the calculated or true values of $K_a$ were small compared to normal for $E_{461D-\beta\text{-galactosidase}}$, about the same as normal for $E_{461G-\beta\text{-galactosidase}}$, and large for the other enzymes.

Table II also shows inhibition by 2-amino-D-galactose and L-ribose. Since $K_a$ values of 40$^{\text{mM}}$ or higher are hard to determine accurately (large concentrations of inhibitor have to be added with only small rate effects) and since they only indicate that binding is very poor, we have not given precise values in those cases. $E_{461D-\beta\text{-galactosidase}}$ was the only enzyme that was inhibited well by 2-aminogalactose (it was actually inhibited better than was the normal enzyme). $E_{461H-\beta\text{-galactosidase}}$ was strongly inhibited. $E_{461K-\beta\text{-galactosidase}}$ was inactivated only $30\%$, whereas $E_{461G-\beta\text{-galactosidase}}$ was inhibited to about the same extent as normal $\beta\text{-galactosidase}$. $E_{461Q-\beta\text{-galactosidase}}$ was strongly inhibited. $E_{461K-\beta\text{-galactosidase}}$ was inactivated only $30\%$, whereas $E_{461G-\beta\text{-galactosidase}}$ was inhibited to about the same extent as normal $\beta\text{-galactosidase}$. $E_{461Q-\beta\text{-galactosidase}}$ was strongly inhibited. $E_{461K-\beta\text{-galactosidase}}$ was inactivated only $30\%$, whereas $E_{461G-\beta\text{-galactosidase}}$ was inhibited to about the same extent as normal $\beta\text{-galactosidase}$.

$\text{pH Profiles}$—Fig. 3 shows the $k_{cat}$ and $K_m$ values for the...
enzymes as functions of pH. β-galactosidase is unstable and hard to assay at low pH even with normal β-galactosidase; and thus, in most cases, the kinetic constants were not evaluated at pH values below 6.0. Note that the $k_{cat}$ values for E461D-β-galactosidase with ONPG increased dramatically as the pH was increased, with a midpoint of ~7.4, but that there was not a similar increase when PNPG was the substrate. The $k_{cat}$ values for E461G-, E461Q-, and E461K-β-galactosidases with ONPG were more or less constant as functions of pH. E461H-β-galactosidase had a high $k_{cat}$ value at pH 6, but the values dropped in a complex manner, with a midpoint of ~7.0. At pH values higher than 8.0, the data with E461H-β-galactosidase represent the activities found after "substrate inactivation" had occurred (as will be described below). For E461G-, E461Q-, and E461K-β-galactosidases, the $K_m$ values increased at pH values less than 7.0, but the values were essentially constant at pH values between 7.0 and 10.0. The $K_m$ values for E461D-β-galactosidase increased somewhat as the pH increased. The $K_m$ values for E461H-β-galactosidase increased and then decreased as the pH was increased.

Fig. 4 shows that the rate of catalysis with E461H-β-galactosidase decreased as a function of time in the presence of substrate at pH 8.0. Fig. 4 also shows that the activity at pH 8.5 was very low even after only 1 min, but this was because the activity in that case dropped very rapidly to low values. This enzyme was, however, stable at high pH because it did not irreversibly lose activity at high pH (8 to 10) in the absence of substrate (enzyme was incubated without substrate at high pH and then assayed at pH 7.0).

Action on Lactose—The enzymes all had very low activities when lactose was the substrate. E461K-β-galactosidase had no activity even though 0.1 mg of enzyme was incubated with 20 mM lactose for 65 h. For the other enzymes, the activities found were 0.0026 unit/mg for E461D-β-galactosidase, 0.0059 unit/mg for E461G-β-galactosidase, 0.0015 unit/mg for E461Q-β-galactosidase, and 0.0007 unit/mg for E461H-β-galactosidase (compared to 28.4 unit/mg for the wild-type enzyme). In the presence of 50 mM lactose, very similar values were obtained. It was not practical to determine $V$ and $K_m$ values with lactose because very large amounts of the enzyme were needed, and the assays required very long incubation times (>60 h). However, the values obtained with 20 mM lactose are essentially equal to $k_3$ for the following two reasons. First, the rates with 50 mM lactose were not very different from those with 20 mM lactose, suggesting that 20 mM was well over the $K_m$ values for the enzymes for lactose; and thus, the rates are equal to $V$. Second, the $k_3$ values for β-galactosidase should be the same for every substrate; and since the $k_3$ values obtained with ONPG and/or PNPG (Table I) were all much higher than the rates found here with lactose, $k_3$ must be rate-determining, and these rates must therefore be equal to $k_3$.

DISCUSSION

It is important, when doing site-directed studies, that several substitutions be made at a position to definitively establish that a particular residue is important for catalysis. A loss of activity when only one substitution is made may be due to a simple conformation change rather than to the fact that the group is essential for catalysis. Also, if the changes in activity that result from a series of substitutions follow some rational pattern, it is easier to determine the role of the residue. Ideally, every possible change should be made, and the effects studied in detail. Obviously, that is not usually practical. The specific substitutions made in this study were chosen both on biochemical considerations and on the basis of preliminary screening (Bader et al., 1988; Cupples and Miller, 1988). The substitution of Asp for Glu-461 introduced another side chain with an identical negative charge. However, Asp is shorter than Glu, and there could be a "space" or "cavity" present. The substitution with Gly removed the entire side chain at the nitrogen of His because the electron densities at the nitrogens of His are greater than those at the oxygens of Gly interacts. Finally, the Lys substitution introduced a positive charge in place of a negative charge. Lysine, of course, also has a longer side chain than has Glu.

The study reported here shows that Glu-461 is highly important for the overall catalytic action of β-galactosidase. All of the enzymes from mutant sources (except E461H-β-galactosidase) had very low activity with each of the substrates studied (<0.3% of normal in even the best case). However, sufficient residual activity remained with each of the enzymes purified from the mutants for us to define the roles of Glu-461 in β-galactosidase.

Physical Characteristics of Enzymes—The normal and substituted enzymes were >95% pure in every case (see Fig. 1). The specific activities of the enzymes isolated from the mutant cells were low therefore because the activity was adversely affected, not because the enzymes were impure. The enzymes from the mutants appeared in the same fractions as normal β-galactosidase throughout purification. This suggests that the overall physical properties which relate to purification (e.g. size, charge, aggregation, etc.) of each of the enzymes from the mutants were similar to those of the wild-type enzyme. Especially important is the fact that the enzymes were all in tetrameric form (all eluted from the Superose-12B size fractionation column at similar positions to the elution position of the wild-type enzyme).

Each of the substituted enzymes was less stable to heat.
Role of Glu-461 in β-Galactosidase (E. coli)

(55 °C) than was the wild-type enzyme. This was not entirely unexpected. The state of the active site of most enzymes is very important in establishing their stability (Yao et al., 1984; Chothia and Lesk, 1985). Despite their instability at 55 °C, the enzymes were all stable for at least 5 months at 4 °C and for at least 1 day at 25 °C (when incubated in the assay buffer).

Substrate and Inhibitor Binding—The enzymes had differing abilities to bind both IPTG (a substrate analog inhibitor) and the substrates. E461D-β-galactosidase bound IPTG and the substrates better than did normal β-galactosidase. E461G-β-galactosidase bound IPTG and the substrates in a more or less comparable manner to the normal enzyme. E461Q-, E461H-, and E461K-β-galactosidases bound these compounds much less well. This is the first time that Glu-461 has been implicated in substrate binding. Gln, His, and Lys are either the same size or larger than Glu, whereas Asp and Gly are smaller. Substrates are often held in a constrained (destabilized) state at enzyme active sites (Jencks, 1975). If the substitution of Asp for Glu creates an extra space or cavity, this may relieve the constraint to a certain extent and allow better binding. Size, however, does not seem to be the only critical factor for binding since Gln is about the same size as Glu; and yet binding is disrupted. Also, Lys is no more disruptive to binding than is Gln despite the considerable difference in size and, of course, charge.

The binding capacity seems to be inversely correlated to the k2 values with both ONPG and PNPG. This implies that some sort of steric effect (e.g. strain, desolvation) is important for the k2 values with these substrates and is mediated in some way by the position occupied by Glu-461.

E461D-galactosidase was the only enzyme from among the mutants which bound 2-aminogalactose (a positively charged transition state analog) well. This strongly implies that the negative charge of Glu-461 is important for binding amino sugars and amino alcohols tightly (Huber and Gaunt, 1982; Legler and Herrchen, 1983) and supports the proposal that one of the roles of Glu-461 is as a counterion for stabilization of a carboxonium ion galactosyl transition state intermediate. It is interesting that E461D-β-galactosidase is so unreactive despite binding 2-aminogalactose so well.

The other inhibitor studied was L-ribose (Huber and Brockbank, 1987). On the whole, the enzymes from the mutant sources bound L-ribose much more poorly than did the wild-type enzyme. The only exception was E461H-β-galactosidase, and it had the highest k2 value (see below). These results mean that Glu-461 must play a role of some kind in destabilizing galactosyl into a planar conformation and that this is important for the hydrolysis (k3) step.

Effect on Galactosylation—The k2 values (representing galactosylation) were all low compared to those of the wild-type enzyme. However, relatively speaking, they were quite large with ONPG, smaller with PNPG, and very small with lactose for every substitution tested. These results are best explained by assuming that Glu-461 is necessary for the acid catalytic component of k2 (in a concerted fashion along with the proton-donating residue). Sinnott and Souchard (1973) and Sinnott et al. (1978) reported that glycosidic cleavage of 3′-galactosidase substrates will occur to a certain extent without acid assistance if the leaving aglycone is acidic (i.e. a good leaving group). Presumably catalytic forces, such as strain or desolvation, are responsible for the nonacidic part of the cleavage in those cases. The aglycone moiety of lactose (glucose) is clearly much more basic than o- or p-nitrophenol; and thus, one would expect that acid catalysis would be (comparatively) a much more important part of the catalysis with lactose (since other effects are unimportant). The results with the substituted enzymes relate to this. The k2 values with ONPG were quite high despite the substitutions for Glu-461 because o-nitrophenol is a good leaving group, and catalytic forces other than acid assistance have significant effects. The k2 values were very low with lactose because acid assistance is highly essential and is poor without Glu-461. With PNPG, factors such as strain or desolvation are probably not as significant as with ONPG.

E461D-β-galactosidase had low k2 values with each substrate tested. Distance differences could be important.

The k2 for E461K-β-galactosidase with lactose as substrate was essentially 0. Lysine, having a positive charge, would probably destabilize the transition state. Therefore, the value of k2 for this enzyme with ONPG and PNPG must be totally due to factors other than acid assistance.

Effect on Degalactosylation—The results showed that Glu-461 is also highly important for the step in which water reacts with the enzyme-galactosyl intermediate to form galactose (hydrolysis or degalactosylation). The k2 value was dramatically lowered as a result of every substitution made except for the substitution with His (the effects on k2 for E461H-β-galactosidase will be discussed separately). The most logical explanation for the effect is that Glu-461 optimally stabilizes a carbonium ion intermediate for reaction with water. The action of Glu-461 in degalactosylation is one of the roles that a carboxyl group at the active site of β-galactosidase was assumed to have (Sinnott, 1978). This, however, is the first hard evidence for this.

Effect of Mg2+—The data indicate that Mg2+ strongly modulates the effect of the substitutions. It is thought that Mg2+ is important in maintaining proper structure at the active site of β-galactosidase and does not play a direct role in catalysis (Case et al., 1973), and it has been proposed that it is important in aligning the proton-donating group for acid catalysis (Sinnott et al., 1978). The data presented here suggest that Mg2+ may also be important in aligning the carboxyl group of Glu-461. A fact that has not been realized before.

Effect of pH—The k2 values for E461G-, E461Q-, and E461K-β-galactosidases were unaffected by pH with ONPG (Fig. 3). This means that the rate-determining step was independent of pH in the range studied. Since the rate-determining step with ONPG is k2 (at least at pH 7), neither general acid nor general base catalysis seems to be involved in k2 for these three enzymes. In the normal enzyme, there is good evidence that general base catalysis is involved in the value of k2 for Glu-461. Since Glu-461 seems to be important for general acid catalysis, the absence of a group that could effectively function in place of Glu-461 should have the effect seen.

The activity of E461D-β-galactosidase with ONPG was strongly dependent upon pH (midpoint was ~8.4). One possible reason could be that the pH may cause a conformational change that shifts the Asp carboxyl group nearer to the carbonium ion; and thus, the rate could increase. Another plausible reason could be that the k2 increase with pH may reflect the ionization of Tyr-503 (Ring et al., 1985, 1988), acting as a general base catalyst in removing a proton from water. The pH profile with PNPG supports this idea. Since k2 is rate determining for PNPG, there should not be an increase because base catalysis is not involved in glycosidic bond cleavage.

E461H-β-galactosidase (Possible Covalent Interaction)—The properties of E461H-β-galactosidase were so different from those of the other enzymes that they deserve separate comment. E461H-β-galactosidase was quite reactive with both
ONPG and PNPG, having a $k_{on}$ value $\sim 6\%$ of the normal value with ONPG and 2% with PNPG. Although the $k_0$ values for this enzyme with these two substrates were high, they were not significantly higher than those for the other substituted enzymes. The real reason for the large $k_{on}$ value with ONPG and PNPG was that this enzyme also has a high $k_0$ value.

The elegant studies of Sinnott (1978) and of Rosenberg and Kirsch (1981) have convincingly shown that a covalent bond forms between the carboxylate group at the active site of normal $\beta$-galactosidase and the galactose of the substrate. We propose that His at position 461 can also react with galactose of substrate; but stability studies at pH 8.0 and 8.5 showed inactivation of E461H-fl-galactosidase at high pH also supported earlier suggestions (Sinnott and Souchard, 1973; Rosenberg and Kirsch, 1981) that $\beta$-galactosidase forms a covalent bond with the galactosyl part of the substrate.

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Scheme I. Depiction of electron trapping ability of $\beta$ imidazoylgalactoside at pH 7.0 and at high pH.
Determination of the roles of Glu-461 in beta-galactosidase (Escherichia coli) using site-specific mutagenesis.
C G Cupples, J H Miller and R E Huber

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