A Highly Reactive β-Galactosidase (Escherichia coli) Resulting from a Substitution of an Aspartic Acid for Gly-794*

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The β-galactosidases of several mutagenized strains of Escherichia coli K12 which grew on lactobionate were found to be heat labile. Sequence analysis of the lacZ gene (ligated into Bluescript®) of one of these strains (E. coli REH4) showed that the only change in the amino acid sequence was a substitution of an Asp for Gly-794. This change caused a dramatic increase in the activity when lactose was the substrate. The \( k_{cat} \) of the purified enzyme from E. coli REH4 (G794D-β-galactosidase) with lactose as the substrate was five to six times as large as the \( k_{cat} \) of the normal enzyme with lactose. Purified G794D-β-galactosidase was, however, less stable to heat and also to chymotrypsin (which cleaves next to Trp-585) than was normal β-galactosidase. G794D-β-Galactosidase bound substrates and substrate analog inhibitors less well than did normal β-galactosidase while planar transition state analog inhibitors were more strongly bound. The ability to bind 2-amino-D-galactose (a positively charged transition state analog inhibitor) was either unaltered or was decreased somewhat. The data showed that the alteration in structure caused an increase in the value of \( k_2 \) (the rate constant for the step in which the glycosidic bond is cleaved) with each substrate tested (the increase was at least 25-fold when lactose was the substrate) while \( k_3 \) was decreased about 4-fold (\( k_3 \) is the rate constant for the common hydrolysis step with each substrate). Since \( k_2 \) is rate determining when lactose is the substrate of the normal enzyme, the increase in \( k_3 \) resulted in a large increase in rate despite the fact that the value of \( k_3 \) decreased. Large rate increases were not found with the other two substrates because the \( k_3 \) values were not increased by large factors and because the decrease in the value of \( k_3 \) negated the effects of the increased \( k_2 \) values. The destabilization of the substrate binding coupled with a stabilization of the binding of a planar transition state is a possible cause of the significant increase in the value of \( k_2 \) and of the enhanced activity with lactose.

β-Galactosidase (EC 3.2.1.23) is the first product of the lac operon of Escherichia coli and is coded for by the lacZ gene. The primary structures of both the protein and the lacZ gene have been determined (Fowler and Zabin, 1978; Kalnins et al., 1983). The natural substrate of the enzyme is lactose, and the enzyme can carry out two reactions (hydrolytic and transgalactosylic) with this substrate (Wallenfels and Weil, 1972; Huber et al., 1976). In the transgalactosylotic reaction, the β-(1→4) linkage is broken and a β-(1→6) linkage is formed to give allolactose (the natural inducer of the lac operon (Muller-Hill et al., 1964)). The enzyme also hydrolyzes other β-galactosides. β-Galactosidase is activated by Na+ or K+ and has a requirement for Mg\(^2+\) or Mn\(^2+\) (Wallenfels and Weil, 1972). Work has shown that the enzyme mechanism proceeds through a galactosyl intermediate which alternates between a carboxylation and a covalently bound form (Sinnott and Souchar, 1973; Rosenberg and Kirsh, 1981; Cupples et al., 1990). The formation of the galactosyl intermediate is probably aided by binding effects (stabilization of the transition state, solvent entropy effects, etc.), but it is thought that acid catalysis also plays a significant role. Studies have shown that Tyr-503 probably acts as a general acid in the first step of the catalysis and later as a general base catalyst (Ring et al., 1985, 1988; Ring and Huber, 1990) while Glu-461 is probably involved in stabilizing the galactosyl intermediate of the enzymatic reaction (Herrchen and Legler, 1984; Bader et al., 1988; Cupples and Miller, 1988; Cupples et al., 1990).

Planar derivatives of β-galactose have been found to be strong competitive inhibitors of β-galactosidase (Lee, 1969; Lehmann and Schroder, 1972; Wentworth and Wolfenden, 1974; Huber and Brockbank, 1987). These planar derivatives probably bind strongly because they resemble the transition state of β-galactosidase. There is evidence that β-galactosidase brings strain and other such forces into play for catalytic efficiency and, thus, if a substrate is such that strain or similar forces can have an effect, the reaction will go more rapidly (Sinnott and Souchar, 1973; Sinnott and Withers, 1974).

Langridge (1968a, 1968b, 1968c, 1968d) and Langridge and Campbell (1968) mutated E. coli and isolated mutants with modulated β-galactosidase activity. Of particular interest for this study were mutations that allowed growth on lactobionic acid. Lactobionic acid does not induce the lac operon and is very poorly metabolized by E. coli. Langridge (1969) was able to obtain three mutants of E. coli which grew on lactobionic acid and which contained β-galactosidase which was less stable to heat than the rest. The β-galactosidases of the three mutants were able to hydrolyze lactose and lactobionic acid at very rapid rates. The enzymes were not purified and the molecular changes which caused the activity changes were not identified.

In this study we report the formation and isolation of mutants similar to those described by Langridge (1969) as well as studies of the properties of a β-galactosidase purified from one of the mutants with greatly enhanced activity with lactose as the substrate. The lacZ gene from the mutant was cloned into Bluescript® and its entire sequence was deter-

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MINERED. THE RESULTS SHOW THAT THE ENHANCED ACTIVITY WITH 
LACTOSE RESULTS FROM A SINGLE SUBSTITUTION (OF GLY-794 BY AN 
ASP) AND IS PROBABLY A RESULT OF DIFFERENT BINDING INTERACTIONS 
WITH THE SUBSTRATE AND WITH A TRANSITION STATE INTERMEDIATE OF 
THE β-GALACTOSIDASE REACTION.

**MATERIALS AND METHODS**

**ENZYMES AND CHEMICALS—RESTRICTION ENDONUCLEASES (NarI, BstBI, 
AccI, HinCII, Rsal, Clal, KpnI, EcoRI, EcoRV, SacI, PstI, BssHII) 
WERE FROM FISHER OR SIMILAR SOURCES, AND THE PUREST GRADES AVAILABLE 
WAS COUNTED IN ORDER TO DETERMINE THE PERCENT SURVIVAL.

**GROWTH CONDITIONS—BACTERIOLOGICAL MEDIA WERE FROM DIFCO. CELL 
PASTEURIZED MEDIUM CONCENTRATED BY FREEZE-DRYING AND 
DISTRIBUTED IN 10-MILLILITER AMOUNTS.

**BACTERIAL STRAINS—E. coli K12, inducible wild-type strain; E. coli 
ML308, constitutive wild-type strain; E. coli B, inducible mutant 
strain (lacZΔ1351) (Ghiorso et al., 1984); Hfr 3000 X74, F Φ C608, Δ (lac)74, rel 
A1, spo T1, thi-1, λ (Beckwith and Singer, 1966).

**GROWTH CONDITIONS—BACTERIOLOGICAL MEDIA WERE FROM DIFCO. 
CELL GROWTH ON LIQUID OR SOLID MEDIA WAS ALWAYS AT 37 °C. AMPICILLIN, 
WHEN USED, WAS AT 100 µg/ml, X-GAL AT 50 µg/ml, AND IPTG AT A FINAL 
CONCENTRATION OF 0.2 mM.

**MUTAGENESIS AND SELECTION—FOR MUTAGENESIS, E. coli K12 WAS 
GROWN TO LOG PHASE IN 200 ML OF M63 MEDIUM. SIX ALIQUOTS OF 30 ML 
EACH WERE CENTRIFUGED TO REMOVE THE MEDIUM AND THEN RESUSPENDED 
in 30 ML OF 0.1 M SODIUM ACETATE BUFFER, pH 5.5. ONE SAMPLE WAS USED 
as a control, and five of the aliquots contained the mutagen, nitro- 
soguanidine (53.6 µg/ml). AT APPROXIMATELY 15-MIN INTERVALS (STARTING 
at 30 MIN), 1 ML OF EACH OF THE SUSPENSIONS WERE MICRO-CENTRIFUGED TO 
REMOVE THE MUTAGEN AND THEN RESUSPENDED IN 1 ML OF M63 MEDIUM. 
FROM EACH OF THESE SUSPENSIONS, 0.1 ML WAS PLATED ON AGAR PLATES 
CONTAINING LB BROTH AND INCUBATED AT 37 °C. THE NUMBER OF COLONIES 
WAS COUNTED IN ORDER TO DETERMINE THE PERCENT SURVIVAL.

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FROM EACH OF THESE SUSPENSIONS, 0.1 ML WAS PLATED ON AGAR PLATES 
CONTAINING LB BROTH AND INCUBATED AT 37 °C. THE NUMBER OF COLONIES 
WAS COUNTED IN ORDER TO DETERMINE THE PERCENT SURVIVAL.

**ALIQUOT FROM EACH OF THE SAMPLES WHICH HAD BEEN EXPOSED TO 
THE MUTAGEN WERE ALSO PLATED ON AGAR PLATES WHICH CONTAINED LACTOBIOTANE 
(0.2% w/v) as a carbon source. MANY COLONIES WERE OBSERVED. IN 
ADDITION, CONTROL MEDIUM (UNTREATED E. coli K12) WAS PLATED TO 
DEMONSTRATE THAT WILD-TYPE E. coli WOULD NOT GROW IN THIS MEDIUM.

**LIQUID CULTURES OF 25 RANDOMLY CHOOSEN COLONIES FROM AN ALIQUOT 
WITH ABOUT 40% SURVIVAL WERE EACH GROWN TO MID-LOG PHASE IN 25 ML OF 
MEDIUM CONTAINING LACTOBIOTANE FOR A CARBON SOURCE. OF THE 25 
SUSPENSIONS, 25 Grew well, AND THESE WERE MICRO-CENTRIFUGED AND RESUS-
PENDED IN pH 7.5 PHOSPHATE BUFFER. THE CELLS WERE BROKEN WITH A 
FRESEIN MILLILITRE OF THE SAMPLING WERE BROUGHT TO EQUAL ACTIVITY 
MILLILITRE BY DIGLUTION WITH THE PHOSPHATE BUFFER, AND A THERMOSTABILITY 
TEST WAS PERFORMED ON THE β-GALACTOSIDASE OF EACH OF THESE 23 MUTANT 
SAMPLES BY FIRST DOING ASSAYS IN TRIPlicate AND THEN PLACING THEM IN 
A WATER BATH AT 55 °C FOR 10 MIN AND ASSAYING IN TRIPlicate AGAIN. THE 
THERMOSTABILITY TEST WAS ALSO DONE ON THE β-GALACTOSIDASE FROM 
THE WILD-TYPE E. coli (ML-308) TREATED IN THE SAME WAY AS WERE THE MUTANT 
CELLS.

**PURIFICATION OF THE β-GALACTOSIDASES—BOTH THE NORMAL β-
GALACTOSIDASE (FROM E. coli ML308) AND THE β-GALACTOSIDASE FROM ONE OF 
THE MUTANT E. coli (NAMED E. coli REH4) WERE PURIFIED BY A PREVIOUSLY 
REPORTED METHOD (KING ET AL., 1985). THE CONCENTRATIONS OF THE 
β-GALACTOSIDASES WERE DETERMINED BY USING THE ESTABLISHED EXTINCTION 
COEFFICIENT OF 2.09 cm⁻¹ mg⁻¹, AN AVERAGE OF THE VALUES REPORTED IN 
THE LITERATURE (WALLENFELD WEIT, 1972).

**ISOLATION AND CLONING OF THE MUTATED lacZ GENE—CHROMOSOMAL 
DNA FROM E. coli REH4 (THE MUTANT SELECTED FOR STUDY) WAS PREPARED 
BY THE METHOD OF THOMAS ET AL. (1966) AND DIGESTED WITH NarⅠ AND 
BstBI WHICH SHOULD YIELD A 3.8-KB FRAGMENT (PREDICTED FROM THE 
SEQUENCE OF THE lac operon FROM E. coli). AFTER 1% AGAROSE GEL 
ELECTROPHORESIS, THE DNA IN THE REGION OF THE GEL WHICH WOULD HAVE
studies, several concentrations of inhibitor were used (some below and some above the $K_v$ value). For routine assay during purification 1 mm ONPG was used.

**Methanol and 2-Mercaptoethanol Effects on β-Galactosidase from E. coli REH4**—Various alcohol and sugar compounds bind to the “galactosyl” form of β-galactosidase and react with the galactosyl component to form a galactosyl adduct (Deschavanne et al., 1978; Huber et al., 1984). If the reaction with the compounds (“acceptors”) is faster than the reaction with water and if the step involving the addition of water (or acceptor) is rate determining, there will be an increase in the overall rate of reaction. For normal β-galactosidase this occurs if ONPG is the substrate (for several acceptors) but not with PNPG because the step involving water (or acceptor) is not rate determining with PNPG. It has also been shown that if one plots $appk_n$ versus $(k_n - appk_w)/[A]$ (where the $appk_w$ values are the $k_n$ values obtained at various concentrations of acceptor and where [A] is the concentration of acceptor), the intercept will be $k_p(k_n + k_q)$ (Deschavanne et al., 1978; Huber and Gaunt, 1982; Huber et al., 1984). With the enzyme from E. coli REH4, an increase in rate was found with methanol (in a parallel experiment using a series of different possible acceptors), and the methanol increased the rate of both ONPG and PNPG hydrolysis (although the effect on ONPG was greater). Therefore, a series of Michaelis analyses with various concentrations of methanol (0.1-1.6 M) were carried out. The data from the intercepts of the $appk_n$ versus $(k_n - appk_w)/[A]$ plots, and the $k_n$ values of reaction of the enzyme with ONPG, PNPG, and lactose allowed us to obtain good estimates of the values of $k_2$ (rate constant for the step in which the glycosidic bond is cleaved) and $k_k$ (rate constant for the hydrolysis step). In order to confirm that the values were correct, the effect of 2-mercaptoethanol (1-100 mM) on the Michaelis kinetics was also tested.

**Assays with Lactose—** Enzyme was incubated in the TES assay buffer with 50 mM lactose. Glycerol (50 mM) was also added to prevent the free glucose product which accumulated in the assay tube from acting as a transgalactosyl acceptor (and, thereby, significantly decreasing the amount of glucose present (Huber et al., 1976)). Aliquots were taken at various times and the amounts of allolactose and of glucose produced/minute were measured by gas chromatography methods which have been described earlier (Huber and Hurlburt, 1986).

**Stability in the Presence of Chymotrypsin—** Two chymotrypsin hydrolysis reactions were set up with the normal enzyme and with the enzyme from E. coli REH4. In the case of each enzyme, one study was done in the absence of IPTG while the other was done in the presence of 50 mM IPTG. The concentration of β-galactosidase in these reaction mixtures was 3 mg/ml while the concentration of chymotrypsin was 0.02 mg/ml. Aliquots were removed periodically and added to a solution of 0.5 mM phenylmethylsulfonyl fluoride at 2 °C which stopped the chymotrypsin reaction. These aliquots were assayed for enzyme activity and samples of the aliquots were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**RESULTS**

**Mutagenesis and Selection—** All of the E. coli K12 cultures which had been treated with nitrosoguanosine showed a significant reduction in colony growth on LB medium. A culture in which the percent survival was near 40% when compared with the untreated control was chosen for isolation of mutants. Samples from that culture were plated on media containing lactobionate. A control culture, which had not been exposed to the mutagen, was also plated to demonstrate that wild-type colonies could not use lactobionate as a carbon source and, indeed, there was no growth of wild-type cells on the lactobionate plates. A very large number of colonies from the mutagenized culture were, however, observed to grow on the lactobionate medium, indicating that mutants had formed. Langridge (1969) showed that mutants such as these, which grow on lactobionate, can arise from two causes. Either more normal enzyme is formed (called quantitative mutants) or the same amount of enzyme is formed, but the enzyme that is present is more active (called qualitative mutants). He was able to select for qualitative mutants by the fact that they were heat sensitive. The same technique was used in this study. Of the 25 mutant colonies, 23 grew well in liquid medium, and a crude homogenate of each of these mutant lines was tested for temperature sensitivity of the β-galactosidase. Six of the 23 crude homogenates had β-galactosidase with reduced temperature stability and two (labeled REH4 and REH6) were particularly sensitive. The average percent activity remaining in the thermostensitivity test for the 23 homogenates was 58.2%. The activity remaining in REH4 was 24.1% and in REH6 was 17%. The REH4 strain was arbitrarily chosen for further study. There was 60.9% activity remaining when wild-type E. coli (ML308) was subjected to the thermostest.

**Purification—** Fig. 2 (unreacted lanes, 0-min reaction times) shows that the purified normal β-galactosidase (from E. coli ML308) and the purified β-galactosidase from E. coli REH4 were >98% pure.

**Kinetics—** Fig. 3 shows Hofstee (1959) plots of the activity of normal β-galactosidase and of the β-galactosidase from E. coli REH4 with PNPG as the substrate. The maximum velocity of the β-galactosidase from E. coli REH4 was a little higher

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**FIG. 2.** Gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of β-galactosidase purified from wild-type E. coli ML308 and of β-galactosidase purified from E. coli REH4 (0-min reaction lanes) and of the same enzymes after treatment for various times with chymotrypsin.

**FIG. 3.** Hofstee (1959) plots ($V_0$ versus $V_0/[S]_0$) of normal β-galactosidase and of β-galactosidase purified from E. coli REH4 with PNPG as the substrate and in the presence and absence of IPTG. ■ — ■, PNPG-normal enzyme; O—-O, PNPG with 0.4 mM IPTG-normal enzyme; □—□, PNPG-enzyme from REH4; △—△, PNPG with 0.4 mM IPTG-enzyme from REH4. The $V_0$ data are represented as observed rate constants (s⁻¹). The [PNPG] concentration is in mM.
than that of the normal enzyme while the $K_m$ value was several-fold higher (note the much steeper slope for the enzyme from *E. coli* REH4). Also shown on the plot are the effects of IPTG on the rates. The graph shows that IPTG inhibits the normal enzyme much more strongly than the $\beta$-galactosidase from *E. coli* REH4 (in relative terms the slope became steeper when IPTG inhibited the normal enzyme than was the case when IPTG inhibited the enzyme from *E. coli* REH4). Table I shows the $k_{cat}$ and $K_m$ values calculated from these plots. Table II gives the $K_m$ values for IPTG and for other inhibitors. The $K_m$ values were used in the studies for $k_{cat}$, because the inhibitor concentration was not limiting. The enzymes from wild-type *E. coli* and the two mutant strains were tested with L-ribose and 2-amino-D-galactopyranose as acceptors.

The rate of glucose production with lactose as the substrate when determined by gas-liquid chromatography was about 33% higher for the enzyme from wild type. Since this ratio is very similar to the rate differences between the enzymes for the hydrolysis of lactose (production of glucose), the rate of transgalactosylis (Huber *et al.*, 1976) must be largely unaffected by the mutation.

The addition of methanol (various concentrations from 0.1 to 1.6 mM) increased the rate of reaction with both ONPG and PNPG as the substrates. The increases with ONPG were greater than they were with PNPG. The values of the intercepts of $k_{cat}$ versus ($k_{cat}$ - $appk_{cat}$)/[A] lines with methanol as acceptor are shown on Table I along with the standard errors of the intercept values. The addition of 2-mercaptoethanol (various concentrations from 1 to 100 mM) caused a decrease of the rates with both substrates and the values of the intercepts of $k_{cat}$ versus ($k_{cat}$ - $appk_{cat}$)/[A] plots for 2-mercaptoethanol are also shown on Table I along with the standard errors of these intercept values. The slopes were fitted by the method of least squares, and in each case (with both substrates and with both acceptors) the regression coefficient ($R$) was greater than 0.95. The enzyme from the mutant was also tested to determine if the methanol or the mercaptoethanol caused any irreversible inactivation. The enzyme (1 mg/ml) was incubated at the highest concentrations of methanol (1.6 mM) and of mercaptoethanol (100 mM) used in the studies for 3 h (a time much longer than the 5-min maximum time used in any of the assays). The enzymes in these high concentrations of acceptor were then diluted 1,000-fold for assay, and they were found to have the same activity that they had at the beginning of the incubation period and the same activity that a control without the presence of the acceptors had. Thus, the acceptors did not cause any irreversible denaturation.

### Table I

|         | $k_{cat}$ | $K_m$ | $k_i$ | $k_o$ | Methanol intercept | Mercaptoethanol intercept |
|---------|-----------|-------|-------|-------|--------------------|--------------------------|
|         |           |       |       |       |                   |                          |
| **ONPG**|           |       |       |       |                   |                          |
| Wild type | 750 ± 30 | 0.12 ± 0.02 | 2,100 | 1,200 | ND                 | ND                       |
| REH4    | 285 ± 15  | 0.16 ± 0.03 | 2,320–2,990 | 315–325 | 640 ± 2 | 92 ± 0.7 |
|         |           |       |       |       | (R = 0.95)         | (R = 0.98)               |
| **PNPG**|           |       |       |       |                   |                          |
| Wild type | 90 ± 5  | 0.030 ± 0.003 | 90 | 1,200 | ND                 | ND                       |
| REH4    | 120 ± 10 | 0.16 ± 0.01 | 190–195 | 315–325 | 190 ± 3 | 66 ± 0.6 |
|         |           |       |       |       | (R = 0.96)         | (R = 1.00)               |
| **Lactose**|           |       |       |       |                   |                          |
| Wild type | 60 ± 10 | 1.4 ± 0.3 | 60 | 1,200 | ND                 | ND                       |
| REH4    | 315 ± 40 | 2.7 ± 0.2 | >1,500 | 315–325 | ND                 | ND                       |

*From Tenu *et al.*, 1971.
mining the thermal stability of the enzyme, and 4) determining the $K_m$ of the $\beta$-galactosidase for PNPG. In every aspect, this testing confirmed that the properties of the enzyme expressed were the same as those from E. coli REH4.

**DNA Sequencing Results**—Two base changes were found in the entire DNA sequence of the lacZ gene from the E. coli REH4 strain. In one of them a G was substituted for an A at position 309 of the lacZ, which corresponds with the third base of the codon for Val-103. This new codon, however, still codes for Val. The important change was a substitution of an A for a G at position 2382 of lacZ, and this codes for Asp when mutated (Edwards et al., 1987) better than does normal $\beta$-galactosidase.

**DISCUSSION**

This report describes the identification and properties of a purified $\beta$-galactosidase with a substitution of an Asp for Gly-794. The substituted enzyme (G794D-$\beta$-galactosidase) has much higher activity than normal for lactose. The kinetic data clearly show that G794D-$\beta$-galactosidase bound substrates and the substrate analog inhibitor IPTG less well than did normal $\beta$-galactosidase while it bound planar transition state analogs (Lee, 1969; Lehmann and Schroter, 1972; Wentworth and Wolfenden, 1974; Huber and Brockbank, 1987) better than does normal $\beta$-galactosidase.

The following is the generally accepted $\beta$-galactosidase mechanism for synthetic nitrophenol substrates in the presence of acceptor molecules.

![Scheme 1](image1)

where $E = \beta$-galactosidase; NPG = nitrophenol substrate; GAL = galactose; A is the acceptor (methanol or 2-mercaptoethanol); dots indicate that a complex of some type is formed. The $k_3$ step is the glycosidic bond cleavage step while the step represented by $k_2$ is the hydrolytic step (addition of water). For this mechanism the values of $k_{cat}$ and $k_m$ are described by the following equations (Tenu et al., 1971).

$$k_{cat} = k_2 k_3 (k_h + k_b)$$
$$K_m = k_2 K_h (k_h + k_b)$$

When $k_2$ is rate determining (as with PNPG for the normal enzyme (Tenu et al., 1971)) the constants become

$$k_{cat} = k_2$$
$$K_m = K_h$$

The following equation has been shown to describe the reaction rates of $\beta$-galactosidase in the presence of an acceptor (Deschavanne et al., 1978; Huber and Gaunt, 1982; Huber et al., 1984).

$$\frac{\text{app} k_{cat}}{[\text{A}]} = \frac{\text{app} k_{cat} k_3 + k_b}{k_2 + k_4} K_h + k_2 k_4 (k_2 + k_4)$$

The $k_i$ value represents the rate at which the acceptor reacts with the galactosyl form of the enzyme. The $k_{cat}$ describes the maximum rate in the absence of acceptor while app$k_{cat}$ is the maximum rate in the presence of acceptor. The intercept of a plot of app$k_{cat}$ versus $(k_{cat} - \text{app} k_{cat})/\text{[A]}$ will be equal to $k_2 k_4 / (k_2 + k_4)$, and it follows (from simple analysis) that both $k_2$ and $k_4$ must have values larger than that of the intercept. In
the same way, each $k_2$ value and also, the $k_5$ value, has to be greater than the $k_{cat}$ values ($k_{cat}/(k_2 + k_5)$) of the substrates involved (ONPG, PNPG, or lactose). Using this information, the following statements can be made. The value of $k_0$ (PNPG) is $>190$ s$^{-1}$ (because $k_0/k_2/(k_2 + k_5)$ with methanol and ONPG was 190 s$^{-1}$). The value of $k_2$(ONPG) is $>640$ s$^{-1}$ (because $k_2/k_5/(k_5 + k_6)$ with methanol and ONPG was 640 s$^{-1}$). The value of $k_3$ is $>315$ s$^{-1}$ (because $k_3/k_6/(k_6 + k_5)$ for lactose was 315 s$^{-1}$). In the case of $k_5$, it should be remembered that $k_5$ has the same value for every substrate because the hydrolysis step is common for each substrate. The $k_{cat}$ for the G794D-β-galactosidase was the largest when lactose was the substrate and it, therefore, follows that the value of $k_5$ has to be higher than the lactose $k_{cat}$. One can go back and refine the values of the rate constants obtained. If $k_0$(PNPG) were exactly 190 s$^{-1}$, the value of $k_0$ would be 325 s$^{-1}$ (i.e. calculated using the fact that the $k_{cat}$ for PNPG is $k_0/k_2/(k_2 + k_5)$ and is equal to 120 s$^{-1}$). Any value of $k_0$(PNPG) higher than 190 s$^{-1}$ would give a lower value for $k_0$. Thus, $k_0 = 315–325$ s$^{-1}$. One can use this range of $k_0$ values to obtain a better estimate of the value of $k_0$(ONPG). If the value of 315 s$^{-1}$ is substituted into the equation for $k_{cat}$ (i.e. $k_{cat} = k_0/k_2/(k_2 + k_5) = 285$ s$^{-1}$), the value for $k_0$(ONPG) works out to be 2,990 s$^{-1}$. If the value of 325 s$^{-1}$ is substituted, the value for $k_0$(ONPG) works out to be 2,320 s$^{-1}$. Thus, $k_0$(ONPG) = 2,320–2,990 s$^{-1}$. Then also, if the lower value for $k_5$ (315 s$^{-1}$) is substituted into the $k_{cat}$ equation for PNPG, a value of 195 s$^{-1}$ for $k_0$(PNPG) is obtained. Any higher $k_0$ value would give a lower value for $k_0$(PNPG). This, taken together with the fact that the $k_0$(PNPG) is $>190$ s$^{-1}$ (as established above) shows that $k_0$(PNPG) = 190–195 s$^{-1}$. The $k_{cat}$(lactose) value is impossible to accurately calculate. However, since the $k_{cat}$ value for lactose is approximately equal to the $k_0$ value, it follows that $k_0$(lactose) must be much greater than $k_5$. A conservative estimate of the lower limit could be 1,500 s$^{-1}$. Thus, $k_0$(lactose) $>1,500$ s$^{-1}$. All of these calculated values are shown on Table 1. Except for the value of $k_0$(lactose) (for which there is only a lower limit), the values all fall within a relatively narrow range. It should, however, be strongly emphasized here that the standard errors involved in the determinations of the $k_{cat}$ and $appk_{cat}$ values (Table 1) were between 5 and 10% and, therefore, these values are undoubtedly less accurate than implied by the short ranges of values. We feel, however, that they are reasonably good estimates of the true values since they were determined without any assumptions as to rate-determining effects. Also, the standard errors of the intercepts with methanol as the acceptor were really quite small (Table 1) and since the estimated values of $k_2$ and $k_5$ depend to a significant extent on the estimated values of those intercepts, the errors involved in them would also be small. In addition, if the two intercepts (with PNPG and with ONPG) of the plots of $appk_{cat}$ versus ($k_{cat}$ – $appk_{cat}$)/[A] with 2-mercaptoethanol as the acceptor are used (66 ± 0.6 s$^{-1}$ for PNPG and 92 ± 0.7 s$^{-1}$ for ONPG) and the range of values for $k_0$(PNPG) and $k_0$(ONPG) are substituted into the equations of the intercepts (i.e. $k_0/k_2/(k_2 + k_5)$), the value for $k_0$(2-mercaptoethanol) works out to 97–99 s$^{-1}$ with PNPG and 94–96 s$^{-1}$ with ONPG. These values compare very well and show that the calculated values of $k_0$(PNPG) and $k_0$(ONPG) are essentially correct.

It should be pointed out here that the data obtained did not result from a denaturing effect that either methanol or mercaptoethanol might have on the enzyme. Even at the highest concentrations of these acceptors used, there was no evidence of denaturation even after a period of 3 h (a much longer period of time than was used in any of the assays).

The data with methanol as the acceptor would not be expected to be as accurate as those with mercaptoethanol because, with methanol, the $K_0$ value was higher than the highest concentration of acceptor used while, with mercaptoethanol, the $K_0$ was in the middle of the concentrations used. This follows from an analysis of the slopes of $appk_{cat}$ versus ($k_{cat}$ – $appk_{cat}$)/[A] plots. These slopes are mathematically equivalent to $K_0/(k_0 + k_5)/(k_2 + k_5)$. If $k_0$ is smaller than $k_5$, the slope should be larger than $k_0$ whereas if $k_3$ is smaller than $k_0$, the slope should be smaller than $K_0$. Actual values of $k_5$ were not obtained in these studies but the relative magnitudes of $k_5$ and $k_0$ were. Since mercaptoethanol slows down the rate of reaction, $k_0$ is smaller than $k_3$ and the slope is, therefore, larger than $K_0$. The slopes for the reactions of mercaptoethanol with ONPG and PNPG were approximately 9 and 12 mM, respectively, and thus, $K_0$ must be less than 9 mM. Concentrations below and well above that level were used in the study with mercaptoethanol. On the other hand, the rate increased as a function of the concentration of methanol and, therefore, $k_0$ with methanol must be higher than $k_0$. So the slope for $k_0$(methanol) is smaller than the $K_0$ value. The values obtained were approximately 1.1 and 1.7 M, respectively, for ONPG and PNPG. Thus, the $K_0$ value must be greater than 1.7 M. The highest concentration of methanol used was 1.6 M (higher concentrations were not used in order to avoid the possibility of irreversible denaturation). Even though the data would not be expected to be as accurate as one would like because only concentrations of methanol lower than the $K_0$ were used, the $R$ value was large (showing good linearity) and the standard error of the intercept was very small. More importantly, the slope was finite indicating $K_0$ has a value of less than infinity. If the $K_0$ value were infinite (an infinite $K_0$ value would indicate that no binding of acceptor is taking place and that the methanol is reacting directly with the galactosyl-enzyme intermediate without binding) the slope would also be infinite (a vertical line would be obtained). There are, however, other possible ways of obtaining a slope with a finite value even if the acceptor is not binding. It is possible that the acceptor is reacting directly with the galactosyl-enzyme complex but that the acceptor, when it is present at high concentrations, also denatures the enzyme. It would, thus, make it appear that the methanol is binding because the total enzyme activity remaining would decrease as the methanol concentration was increased. This is a definite theoretical possibility considering the high concentrations of methanol used here. However, the enzyme was incubated for 3 h with the highest concentrations of acceptors used and was found not to have lost any activity upon dilution, and this essentially rules out the possibility that denaturation is the reason for the finite slope. Another possible explanation of the results is that the rate increase found is solvent related and that the methanol only acted by changing the structure of G794D-β-galactosidase (making it more active). This has been ruled out by gas-liquid chromatograph studies in our laboratory which showed that methyl-galactose is formed as a product in these reactions and that the amount formed depends on the concentration of methanol added in exactly the same way as does the rate of reaction.

If $k_0$ is rate limiting, the value of $K_0$ will be essentially equal to $K_0$. (Tenu et al., 1971). Although $k_0$ was not the sole rate-limiting step for any of the substrates, $k_0$(PNPG) was lower than the value of $k_0$ while $k_0$(ONPG) was substantially higher than $k_0$. Thus, it would be expected that changes in binding would be reflected much more in the $K_0$ values of PNPG than in the changes in the $K_0$ values of ONPG. The $K_0$ of PNPG is 4-fold higher in the β-galactosidase from the
mutant than it is with normal enzyme but only about 33% higher with ONPG as the substrate (this was, however, within experimental error of the $K_v$ value for normal enzyme despite being 33% higher in value). Over and above this, the fact that the increase in $K_v$ for PNPG is somewhat equivalent to the increases in the $K_v$ values of IPTG and lactose is a further indication that the values of $k_2$ and $k_b$ which were determined above are correct.

Analysis of the $k_b$ data shows that the value was increased as a result of the mutation for every substrate tested. The value with ONPG had the smallest relative increase. For PNPG the $k_v$ value was essentially doubled in G794D-β-galactosidase as compared with normal β-galactosidase. The most dramatic effect of the substitution was with lactose. There was an increase in the $k_v$ value of at least 25-fold.

The $k_b$ was about 4-fold lower for G794D-β-galactosidase than for normal β-galactosidase, and this accounts for the fact that the $k_{cat}$ with ONPG was smaller for G794D-β-galactosidase than for normal β-galactosidase even though the $k_v$ value was higher. In the case of lactose the $k_b$ value was at least 25-fold greater, but the $k_{cat}$ was only 5-6-fold higher.

The reason for this is that the $k_v$ value is rate limiting in G794D-β-galactosidase with lactose as the substrate and the overall increase is, therefore, limited by the $k_b$ value of G794D-β-galactosidase.

Binding destabilization for the G794D-β-galactosidase (compared with the normal enzyme) occurred for each substrate and substrate analog inhibitor tested (ONPG, PNPG, lactose, IPTG) as noted by the increases in the $K_m$ and $K_v$ values. The binding of each of the two planar transition state analog inhibitors tested (β-galactal and L-ribose), on the other hand, was stabilized by the enzyme from the mutant. (β-Galactal is also a poor substrate of β-galactosidase, but when its inhibitory ability is measured it approximates the binding of a transition state intermediate (Huber and Brockhenk, 1987).) The destabilization of substrate binding and a stabilization of binding of a planar transition state is probably responsible for the increase in $k_b$ for every substrate (when G794D-β-galactosidase was compared with the normal enzyme) even though the increase in $k_b$ was not of the same magnitude for each substrate. Such a result is not unexpected since strain (or another similar effect) does not always result in an equal increase in rate (Deneckes, 1975) because the product of such effects depends upon the structure of the substrate. It is thought that strain (or another similar effect) plays a greater role with some substrates of β-galactosidase than with others (Sinnott and Souchard, 1973; Sinnott and Withers, 1974). Overall, these studies strongly support the theory that strain (or other similar interactions) are important in β-galactosidase action in general and that, in this specific case, they are responsible for the rate differences found.

The finding that $k_b$ decreased as a result of the mutation is not surprising since the tighter binding of the transition state could cause a difference in positioning of the intermediates and, if precise positioning is highly important for hydrolysis, one could expect the $k_b$ value to be decreased.

Contrary to what might be expected for a change of this type, where an increase in activity results from the introduction of a negative charge in place of a residue with no charge, the binding of 2-amino-β-D-galactopyranose appears to be unaffected. 2-Amino-β-D-galactopyranose and other amino sugars that are similar to β-galactose bind tightly to β-galactosidase because of the negative charge (Glu-461) present at the active site (Huber and Gaunt, 1982; Legler and Herrchen, 1983; Cupples et al., 1989). The extra negative charge which results from the new Asp here does not appear to increase the binding ability of this positively charged inhibitor, showing that the charge must be far enough removed from the active site so that it does not influence the binding of this inhibitor. However, if this inhibitor binds essentially as the substrate does, one would expect that its binding would be weakened in the enzyme from the mutant (as occurred with the substrate and with IPTG). Since there was no change in the binding capacity, there is a possibility that the substitution of Asp at position 794 does increase the binding capacity.

The fact that chymotrypsin hydrolyzes a peptide bond (at the carboxyl side of Trp-585) at a more rapid rate with G794D-β-galactosidase than with normal enzyme, indicates that the mutation at position 794 has, in some way, made the bond cleaved by chymotrypsin more accessible. Susceptibility of protein structures to cleavage by proteases is a highly sensitive method of determining changes in protein conformation and, thus, the region around Gly-794 must somehow affect the conformation around Trp-585. Other studies have shown that Tyr-50 at the active site (Ring and Huber, 1989) is also linked to the region around Trp-585 and it is logical, therefore, to suggest that Gly-794 may be located near to the active site.

The decreased stability of G794D-β-galactosidase to heat (relative to the normal β-galactosidase) is also an indication that Gly-794 could be near to the active site. Indeed, the fact that binding is so dramatically affected is also, of course, strong evidence that the group is near to the active site.

The substitution of an Asp for a Gly at position 794 introduces a carboxyl group into an area of β-galactosidase which is already rich in carboxyl groups.

Highly Reactive β-Galactosidase

It is possible that this negatively charged area of the enzyme is involved in binding Mg$^+$, Mn$^{2+}$, Na$^+$, or K$^+$. Alternatively (since the area around Gly-794 conforms to a predicted β-sheet structure, as noted by calculations of the Chou and Fasman (1974a, 1974b) type), it may be involved in a salt linkage with some other part of the β-galactosidase structure which is positively charged. If this sequence were in a β-sheet structure the carboxyls would mainly point in the same direction and could, thus, interact with some area having positive charges (e.g. another β-sheet with alternating positive charges or a positive side of an α-helix).

It is of interest that the substitution is located in a region of β-galactosidase that Langridge and Campbell (1968) and Langridge (1968a) thought, on the basis of their genetic studies, might be important for substrate binding.

Normal β-galactosidase may represent a compromise in evolution where activity with lactose and stability of the enzyme are optimized with respect to each other. Thus, although an enzyme which is much more reactive toward lactose could have evolved, it would have been less stable.

The hydrolysis of the lactose in milk or whey is important commercially because of the lactose intolerance experienced by some individuals and because of problems of industrial disposal of whey. Since the lactose of milk or whey could be more efficiently hydrolyzed by this enzyme and thus less enzyme would be needed for the process, this highly reactive enzyme could be of biotechnological significance.

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Highly Reactive β-Galactosidase

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