Effects of Amino Acid Substitutions at the Active Site in *Escherichia coli* β-Galactosidase

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ABSTRACT

Forty-nine amino acid substitutions were made at four positions in the *Escherichia coli* enzyme β-galactosidase; three of the four targeted amino acids are thought to be part of the active site. Many of the substitutions were made by converting the appropriate codon in *lacZ* to an amber codon, and using one of 12 suppressor strains to introduce the replacement amino acid. Glu-461 and Tyr-503 were replaced, independently, with 13 amino acids. All 26 of the strains containing mutant enzymes are Lac^-. Enzyme activity is reduced to less than 10% of wild type by substitutions at Glu-461 and to less than 1% of wild type by substitutions at Tyr-503. Many of the mutant enzymes have less than 0.1% wild-type activity. His-464 and Met-3 were replaced with 11 and 12 amino acids, respectively. Strains containing any one of these mutant proteins are Lac^+. The results support previous evidence that Glu-461 and Tyr-503 are essential for catalysis, and suggest that His-464 is not part of the active site. Site-directed mutagenesis was facilitated by construction of an f1 bacteriophage containing the complete *lacZ* gene on a single *Eco*RI fragment.

β-GALACTOSIDASE (EC 3.2.1.23) is produced in *Escherichia coli* by the *lacZ* gene, one of the four protein-coding genes which comprise the lactose (*lac*) operon (see review by MILLER and REZNIKOFF 1978). The enzyme catalyzes the conversion of lactose into glucose and galactose, allowing the organism to use lactose as a carbon source. It is a multimeric enzyme, composed of four identical subunits. Despite the fact that each of the subunits has an independent catalytic site, only the tetramer is biologically active (reviewed by ZABIN and FOWLER 1978).

The large size of the individual polypeptide chains (1023 amino acids) may account for the fact that the enzyme is very tolerant of changes in the primary sequence. LANGRIDGE (1974) selected nitrosoguanidine-induced mutants which had less than 50% of wild-type β-galactosidase activity and showed that 72% of those attributable to point mutations were nonsense rather than missense mutations. Similarly, 93% of the point mutations in *lacZ* which prevented growth on lactose were nonsense mutations (LANGRIDGE and CAMPBELL 1969). These studies suggest that most missense mutations have little effect on β-galactosidase activity.

This hypothesis is supported by two studies on the effects of amino acid substitutions in β-galactosidase. WELPLY, FOWLER and ZABIN (1981) used nonsense suppression to produce amino acid substitutions at four sites in the α portion of the molecule, a region known to be involved in tetramer formation (reviewed by ZABIN 1982). They found little or no decrease in enzyme activity. In a previous study (C. G. CUPPLES and J. H. MILLER, unpublished data), we used ethylmethane sulfonate to individually convert 43 glutamine (CAG) and 39 tryptophan (TGG) residues throughout *lacZ* to amber (TAG) codons. Nonsense suppressor strains were used to make 12 different amino acid substitutions at each of these 82 amber codons. Eighty-one of these residues could accept any substitution with minimal change in enzyme activity.

Since random alterations in the primary structure of β-galactosidase have so little effect on enzyme activity, we decided to specifically change amino acids which are thought to be part of the active site. In this paper, we report on the effects of amino acid substitutions at three such residues. Two of the amino acids, the glutamate residue at position 461 and the tyrosine at 503, are probably involved in the catalytic reaction (HERRCHEN and LEGLER, 1984; SINNOTT and SMITH 1978; FOWLER *et al.* 1978). LEGLER and HERRCHEN (1983) and HERRCHEN and LEGLER (1984) have suggested that a third residue, the histidine at position 464, may be involved in substrate binding. For comparison, we have also made substitutions at methionine 3, an amino acid which is in a part of the molecule that is nonessential for enzyme activity (MÜLLER-HILL and KANIA 1974; BRAKE *et al.* 1978).

MATERIALS AND METHODS

Bacterial strains are listed in Table 1. Plasmids are maintained in p90c, phage in JM801 and episomes in s90c. Media have been described by MILLER (1972). All recombinant DNA techniques are standard (MANIATIS, FRITSCH and SAMBROOK 1982; DAVIS, DIBNER and BATTEY 1986) unless

TABLE 1

Bacterial strains

Strain	Sex	Genotype
P90c	F⁻	*ara* Δ(*lacproB*) *thi*
S90c	F⁻	*ara* Δ(*lacproB*) *thi rpsL*
JM801	F⁺*kan*	*ara* Δ(*lacproB*) *thi rpsL*
XA101	F⁻	*ara* Δ(*lacproB*) *gyrA metB argE*⁻*am rpoB supD thi*
XA102	F⁻	*ara* Δ(*lacproB*) *gyrA metB argE*⁻*am rpoB supE thi*
XA103	F⁻	*ara* Δ(*lacproB*) *gyrA metB argE*⁻*am rpoB supF thi*
XA105	F⁻	*ara* Δ(*lacproB*) *gyrA metB argE*⁻*am rpoB supG thi*
XA96	F⁻	*ara* Δ(*lacproB*) *gyrA argE*⁻*am rpoB thi supP*
JM802	*F'lacI proA*⁺, *B*⁺	Δ(*lacproB*) *supE thi*
CSH 13-20a	F'*lacZ proA*⁺, *B*⁺	Δ(*lacproB*) *supE thi* contain progressively larger deletions in *lacZ*

otherwise specified. SDS-polyacrylamide gel electrophoresis of bacterial proteins is described by SILHAVY, BERMAN and ENQUIST (1984). *In vitro* β-galactosidase assays were done using permeabilized whole cells, as described by MILLER (1972).

Galactosides were purchased from Bachem (Torrance, CA), and DNA restriction and modification enzymes and linkers from Pharmacia or IBI. Plasmids pBR322 and pBR329 were obtained from New England Biolabs.

Synthetic suppressors: In addition to the standard suppressor strains (XA96, XA101-XA105) we used seven additional suppressor strains, in which the mutant tRNA is carried on a multicopy plasmid. Details of the construction and use of these suppressors, with comprehensive documentation of specificity and efficiency have been published (NORMANLY *et al.* 1986) or will be published elsewhere. Briefly, it has been established that each suppressor inserts the correct amino acid at least 90% of the time. Efficiency of amber suppression varies between 5% and 100% and is dependent in part on the site being suppressed.

Plasmid and phage construction: To provide a template for oligonucleotide-directed mutagenesis, the entire *lacZ* gene was cloned into an f1 bacteriophage derivative, R229 (BOEKE 1981). Figure 1 illustrates the steps involved in the construction of this phage, f1-Z. The *Pst*I/*Eco*RI fragment which contains the *lacI* gene and part of the *lacZ* gene was subcloned from the plasmid pGM11 (GALAS, CALOS and MILLER 1980). The six oligonucleotides used to construct the missing 53 bp of *lacZ* were synthesized, purified, phosphorylated and annealed as described previously (NORMANLY *et al.* 1986). To introduce an *Eco*RI site 5′ of *lacZ*, the *Hpa*I site in *lacI* was converted into an *Eco*RI site by the addition of *Eco*RI linkers. Since the *lacZ* gene also contains two *Hpa*I sites, the following conditions were used to generate partially digested DNA. One μg of pCCLac4 DNA was digested with 1 unit of *Hpa*I at 37° in 100 μl. Aliquots were withdrawn after 5, 10, 15, 20 and 30 min. The reaction was terminated by extracting the DNA with phenol/chloroform, and precipitating it with ethanol. To add the linkers, the five aliquots were pooled and ligated overnight to phosphorylated *Eco*RI linkers at a molar ratio of 1:50. The ligase was inactivated at 65° for 10 min and the salt conditions were adjusted to 100 mM Tris, 50 mM NaCl and 5 mM $MgCl_2$ (pH 7.4). Excess *Eco*RI was added and digestion was continued at 37° overnight. DNA was extracted with phenol/chloroform and ethanol precipitated before ligation into the *Eco*RI site of R229. Phage containing the complete *lacZ* gene, without *lacI*, were blue on LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal).

The *lacZ*-containing insert was cloned in both orientations into R229, so that single stranded template DNA could be prepared from both strands.

Mutagenesis: Site-directed mutagenesis was done by a variation of the technique of ZOLLER and SMITH (1982), using two primers. The mutagenic and secondary primers are shown in Table 2. (Note that the Met-3 amber mutation was made on the opposite strand from the mutations at the other three sites). DNA from the mutagenesis reaction was used to transform competent JM801 cells. Cells containing phage with missense or nonsense mutations in *lacZ* formed pale blue or white plaques on plates containing Xgal. Single- and double-stranded DNA were isolated from these plaques for sequencing and subcloning.

Transferring mutations from phage to episome: Each mutant *lacZ* gene was recombined onto an episome via a plasmid intermediate. Double-stranded DNA from mutant phage was digested with *Eco*RI and the fragment containing the *lacZ* gene was subcloned into pBR329. Plasmid-containing cells were mated with cells which contained a *lacI*⁻/*lacZ*⁺ episome, and progeny were selected on minimal glucose plates containing tetracycline. Cells were grown overnight in LB medium containing tetracycline to allow recombination between the episomal and plasmid *lacZ* genes. They were then crossed with s90c in order to separate episomes from plasmids. The progeny were selected on minimal glucose plates containing streptomycin and Xgal. Cells containing an episome with a mutation in *lacZ* were white or pale blue.

Analysis: Episomes were transferred to deletion mapping strains (CSH 13 to 20a) by plate mating techniques (MILLER 1972). Finer mapping was done using an additional set of 22 deletion strains. Similarly, episomes which contain an amber mutation were transferred to suppressor strains for analysis. β-Galactosidase activities were assayed *in vivo* on indicator plates containing Xgal. Enzyme levels were determined *in vitro* using *o*-nitrophenyl-β-D-galactoside (ONPG) as a substrate. Cells were assayed for the ability to grow on minimal lactose plates.

RESULTS

Construction of the f1-Z phage: To produce single stranded template for site-directed mutagenesis the *lacZ* gene was cloned into R229, a filamentous M13 phage derivative which contains a single *Eco*RI site in the intergenic region (BOEKE 1981). Cloning *lacZ* as an *Eco*RI fragment required that the single, internal *Eco*RI site close to the 3′ end of the gene be removed, and that new *Eco*RI sites be inserted on either side of the gene. This construction is shown in Figure 1.

LacI (the *lac* repressor gene) and most of *lacZ* were initially cloned as a *Pst*I/*Eco*RI fragment (pCClac1 in Figure 1). The missing portion of the *lacZ* gene was synthesized *de novo* as a small fragment which could be ligated into the *Eco*RI site of pCClac1. The *Eco*RI sites were not reformed during ligation; however the synthetic fragment itself contained an *Eco*RI site im-

TABLE 2

Mutagenic and secondary primers

A. Mutagenic primers:[a]	
Methionine 3	
Amber primer	5′-ATGACCTAGATTACGGAT-3′
Wild type	3′-TACTGGTACTAATGCCTAAGTGACCGGCAG-5′
Glutamate 461	
Wild type	5′-ATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCAC-3′
Basic primer	3′-GCGACCCCTTA***TCACCGGTGCCGCG-5′
Histidine 464	
Wild type	5′-CTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCG-3′
Amber primer	3′-CTTAGTCCGATCCCGCGATTAG-5′
Tyrosine 503	
Wild type	5′-ATTATTTGCCCGATGTACGCGCGCGTGGATGAAGACCAGCCC-3′
Basic primer	3′-CGGGCTAC***CGCGCGCACCTCCTTCTG-5′
B. Secondary primers:[b]	
Z-COOH-4	3′-ATGGTAATGGTCAACCAGACC-5′
Z-COOH-5	3′-GTCTGGTGTCAAAAATAATAAGAATTCTGCAGT-5′

[a] Sequence of the oligonucleotides used to introduce amber and missense mutations at Met-3, Glu-461, His-464 and Tyr-503. Each oligonucleotide is shown with its complementary wild-type strand. Note that the sequence of the Met-3 primer is that of the sense strand. Changes from the wild type are underlined. The mutations at Glu-461 and Tyr-503 are indicated by *** since both nonsense and missense codons were introduced at these sites.

[b] Sequence of the secondary primers. The top one was used in conjunction with the Glu-461, His-464 and Tyr-503 mutant oligonucleotides. The bottom one was used in the construction of the amber mutation at Met-3. Both oligonucleotides are from the set of six used to reconstruct the 5′ end of *lacZ* (see Figure 1).

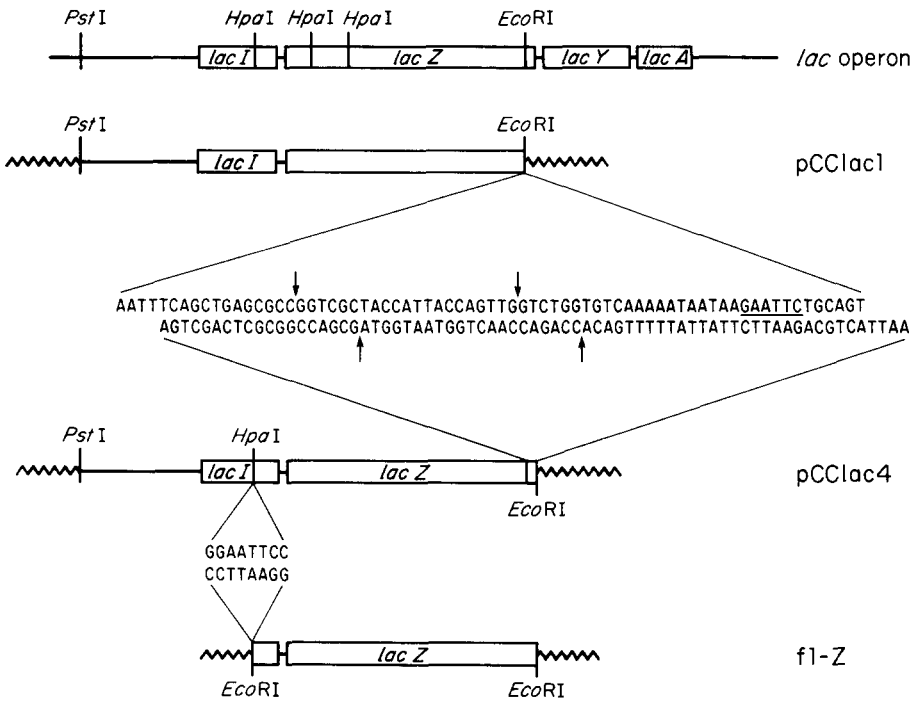

FIGURE 1.—Plasmid and phage constructions. Vector sequences are shown by wavy lines, genes or parts of genes by boxes and intergenic regions by straight lines. The top diagram is a schematic map of the *lac* operon showing the relative positions of the restriction sites used in the constructions. pCClac1 was made by cloning a *PstI*/*Eco*RI fragment containing *lacI* and 98% of *lacZ* into the *Pst*I and *Eco*RI sites of pBR322. The *Eco*RI site is within *lacZ*. The missing portion of *lacZ* was synthesized *de novo* from six oligonucleotides. The sequence also contained a new *Eco*RI site (underlined) immediately 3′ of the *lacZ* termination codons. The boundaries of the individual oligonucleotides are indicated by vertical arrows. The synthetic double stranded fragment was ligated into the *Eco*RI site of pCClac1 to produce pCClac4. The *Eco*RI sites were not reformed. The *Hpa*I site in the 3′ end of *lacI* was converted into an *Eco*RI site by the addition of *Eco*RI linkers. Finally, f1Z was produced by subcloning the pCClac4 *Eco*RI fragment containing the complete *lacZ* and small fragment of *lacI* into the *Eco*RI site of R229.

mediately 3′ of the termination codons. pCClac4 contains the complete *lacI* and *lacZ* genes.

The second flanking *Eco*RI site was introduced into the *lacI* gene, a step which also detected 80% of the *lac* repressor gene and allowed constitutive expression of *lacZ*. The site was produced by converting the *Hpa*I site in *lacI* into an *Eco*RI site, using *Eco*RI linkers. With *Eco*RI sites on either side of *lacZ*, the gene could then be ligated into the *Eco*RI site of R229. The *lacI*$^-$/*lacZ*$^+$ phage produced dark blue plaques on plates containing Xgal. This bacteriophage, f1-Z, has two advantages over the M13 series for these experiments, β-galactosidase expression is constitutive, and the entire *lacZ* gene is on a single fragment, thereby avoiding the complications of alpha complementation.

Production and identification of mutants: Single-stranded f1-Z DNA was used as a template for site-directed mutagenesis. Four oligonucleotides (Table

2B) introduced amber mutations at Met-3, Glu-461, His-464 or Tyr-503. Fourteen additional oligonucleotides introduced missense mutations at Glu-461 or Tyr-503. The efficiency of amber suppression is influenced by the identity of the base immediately 3′ of the amber codon (MILLER and ALBERTINI 1983; BOSSI 1983). With a few exceptions, A and G provide a better suppression context than U or C. Therefore the oligonucleotide which changed Glu-461 (GAA) to an amber codon (TAG) also changed the serine 462 codon from TCA to AGT. For consistency, the same change was also made by all of the oligonucleotides which introduced missense mutations at Glu-461.

Mutant phage formed white or pale blue plaques on plates containing Xgal, and DNA from these phage was sequenced to confirm that the mutation was present. Phage were also checked, as follows, to ensure that there were no secondary mutations. Those containing nonsense mutations produced bright blue plaques when introduced into the suppressor strain which inserted the wild type amino acid. The missense mutations were reverted to the original codon by site-directed mutagenesis, and the resulting phage produced bright blue plaques. The mutant *lacZ* genes were recombined onto an episome via a plasmid intermediate. Once on the episome, each mutation was checked to ensure that it mapped to the correct deletion interval.

The original *lacZ* gene cloned from pGM11 has a weaker promoter (L8), whereas the episome has a strong promoter (P^+). Recombinants containing the P^+ promoter have approximately 15-fold higher levels of β-galactosidase, increasing the sensitivity of subsequent enzyme assays.

Effect of amino acid substitutions at His-464 and Met-3: Table 3 shows the units of β-galactosidase produced by enzymes with twelve individual amino acid substitutions at His-464. The results are compared to those obtained from the same amino acid substitutions at Met-3. All of the substitutions at each site were made by nonsense suppression. The pattern of enzyme activity is similar at the two sites. All 24 strains grew on minimal lactose plates and produced medium to dark blue colonies on plates containing Xgal after 24 h (Figure 2A). When the plates were examined after a shorter incubation, the intensity of the color was clearly proportional to the units of enzyme activity determined *in vitro*.

Effect of amino acid substitutions at Glu-461 and Tyr-503: Thirteen different amino acids were substituted, independently, for the glutamate at position 461 and the tyrosine at 503. At each site, 2 substitutions were made by missense mutation, 6 by nonsense suppression and 5 by both methods. The wild-type amino acids, glutamate and tyrosine respectively, were also reintroduced into the sites by nonsense suppression. Table 4 (Glu-461) and Table 5 (Tyr-503) show the *in vitro* activity of the mutant enzymes, as measured by the hydrolysis of ONPG. The units of enzyme activity of the suppressed nonsense mutations are also shown normalized for suppressor efficiency, so that activity can be compared directly to that of the corresponding missense mutations. In most cases, the activity of the missense and suppressed nonsense mutants differ by less than a factor of 3. In contrast, the activity of most of the mutant enzymes was 2 to 4 orders of magnitude below that of the wild type enzyme. None of the mutant proteins has sufficient activity to allow the cells to use lactose as a carbon source (Lac^-).

TABLE 3

Units of β-galactosidase produced by nonsense suppression of amber mutations at Met-3 and His-464

Amino acid	Met-3	His-464
Ser	719	480
Gln	704	260
Tyr	1263	964
Lys	777	621
Leu	2762	2037
Ala	2581	2167
Cys	2143	2317
Glu	2947	2177
Gly	2342	1224
His	2562	2149
Phe	4316	3964
Pro	1080	1623
Wild type	4200	

Assays were done using permeabilized whole cells, and units of activity were calculated as described by MILLER (1972). The strains are listed in the same order as they appear on the Xgal plates (Figure 2A).

The drastic decreases in enzyme activity cannot be accounted for by increased degradation of the mutant proteins. Protein extracts from wild-type and mutant cells were analyzed by SDS-polyacrylamide gel electrophoresis; the amount of β-galactosidase was similar in all strains (data not shown).

Enzyme activity was also monitored *in vivo* by the intensity of the blue color produced by colonies growing on plates containing Xgal. Figure 2B shows the results for substitutions at Glu 461. Of the suppressed amber derivatives, only the glutamate and histidine substitutions produce a blue colony; the others remain white even after prolonged incubation. The histidine, glycine and aspartate missense mutants are pale blue, and the other missense mutants are white. Figure 2C shows the results for the Tyr-503 mutants. In contrast, to the Glu-461 results, all of the cells containing Tyr-503 mutant enzymes produce blue colonies on Xgal plates after 24 hr of incubation.

DISCUSSION

We have made a total of 37 amino acid substitutions at three residues in *E. coli* enzyme β-galactosidase

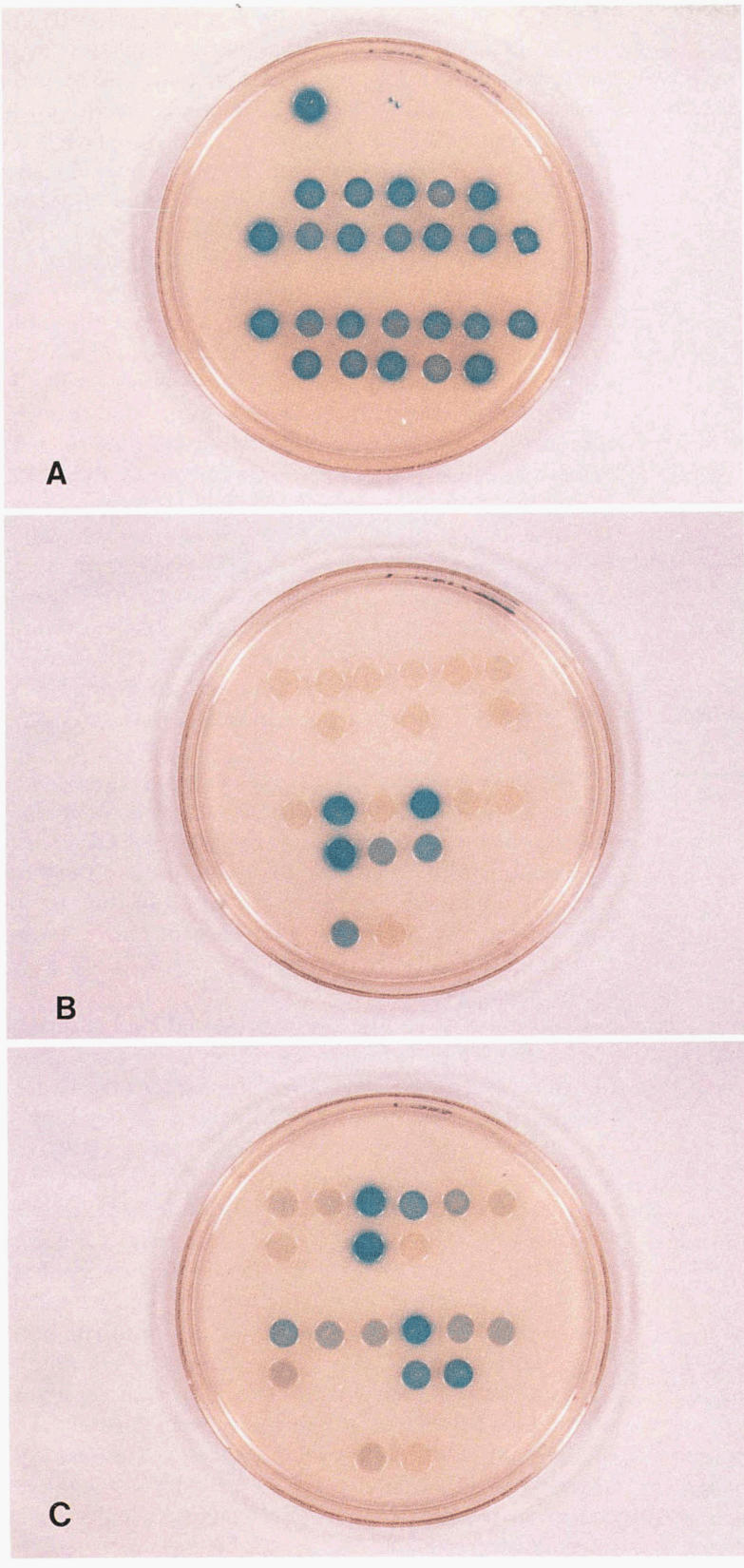

FIGURE 2.—β-galactosidase activity on plates containing Xgal, after 24 hr of incubation. A, Met-3 amber mutation (rows 2 and 3) and His-464 mutation (rows 4 and 5) in the following suppressor strains: row 2 and 5: serine, glutamine, tyrosine, lysine and leucine; rows 3 and 4: alanine, cysteine, glutamate, glycine, histidine, phenylalanine and proline. The wild-type strain is in row 1. B, Glu-461: Colonies in rows 1 and 3 contain the amber mutation in the suppressor strains. The inserted amino acids in row 1 are: serine, glutamine, tyrosine, lysine, leucine and alanine; in row 2: cysteine, glutamate, glycine, histidine, phenylalanine and proline. The missense mutants, or wild type, are directly underneath the appropriate suppressed amber (rows 2 and 4). Row 5 contains the asparate and valine missense mutants. C, Tyr 503: Colonies in rows 1 and 3 contain the amber mutation in the suppressor strains. The order is the same as in B. The missense mutations, or wild-type (rows 2 and 4) are underneath the appropriate suppressor strain. Row 5 contains the asparagine and isoleucine missense mutants.

which have been postulated to be part of the active site. Glu-461 and Tyr-503 are thought to be directly involved in catalysis (HERRCHEN and LEGLER 1984; FOWLER *et al.* 1978; SINNOTT and SMITH 1978). His-464 is not directly involved in catalysis, but LEGLER and HERRCHEN (1983) have suggested that it may be

TABLE 4

Units of β-galactosidase produced by the mutants with amino acid substitutions at Glu-461

Amino acid	Suppressed amber		Missense (or wild type)
	Absolute	Normalized	
Ser	4.7	28.2	—[a]
Gln	0.23	1.41	4.3
Tyr	0.06	0.21	—
Lys	1.2	6.67	0.025
Leu	3.0	4.69	—
Ala	1.8	3.01	27.8
Cys	4.7	9.47	—
Glu	1664	2437	3217 (wild type)
Gly	11.8	21.7	42.6
His	99.8	168	281
Phe	0.31	0.31	—
Pro	0.05	0.20	—
Asp	—	—	1.04
Val	—	—	0.45

[a] — = missense mutation was not made or there was no amber suppressor available to insert that amino acid. The strains are listed in the same order as they appear on the Xgal plates (Figure 2B).

TABLE 5

Units of β-galactosidase produced by the mutants with amino acid substitutions at Tyr-503

Amino acid	Suppressed amber		Missense (or wild type)
	Absolute	Normalized	
Ser	0.09	0.7	0.39
Gln	0.096	1.5	—[a]
Tyr	659	2710	4073 (wild type)
Lys	4.2	26.8	0.3
Leu	0.52	1.0	—
Ala	0.17	0.3	—
Cys	10.2	17.5	0.53
Glu	0.25	0.5	—
Gly	0.42	1.4	—
His	4.9	9.0	4.9
Phe	0.55	0.55	4.32
Pro	1.47	3.6	—
Asn	—	—	0.45
Ile	—	—	0.18

[a] — = missense mutation was not made or there was no amber suppressor available to insert that amino acid. The strains are listed in the same order as they appear on the Xgal plates (Figure 2C).

part of the active site. For comparison, we have also made 12 amino acid substitutions at Met-3, a residue which is not necessary for enzyme activity (MÜLLER-HILL and KANIA, 1974; BRAKE *et al.* 1978).

Predicting the probable effects of amino acid substitutions at Glu-461, His-464 and Tyr-503 on biological activity was complicated by the fact that there is no crystallographic data available for β-galactosidase. Therefore, in order to make as many mutant proteins as possible, we made use of bacterial nonsense suppressor strains to individually insert twelve amino acids at each site. This eliminated the necessity of producing individual missense mutations for each substitution. Simply changing the original codon to an amber (TAG) codon and expressing the gene in a variety of amber suppressor strains allowed the production of a family of mutant proteins, each with a different amino acid substitution. This approach to protein engineering has been employed previously, using five of the naturally occurring amber suppressors (MILLER *et al.* 1979; SMITH *et al.* 1986). The addition of seven synthetic suppressors allowed us to make 12 amino acid substitutions at each site.

Methionine 3: Since Met-3 is in a part of the molecule not required for enzyme activity (MÜLLER-HILL and KANIA 1974; BRAKE *et al.* 1978), it can probably be replaced by any amino acid without significantly affecting enzyme activity. The activity of mutant proteins made using the 12 suppressor strains should, therefore, reflect suppressor efficiencies. As expected, the results shown in Table 3 are comparable with previous data on relative suppressor efficiency (MILLER and ALBERTINI 1983; NORMANLY *et al.* 1986; L. G. KLEINA and J. H. MILLER, unpublished data). We therefore used the percentage of wild-type activity at this site to normalize the units of enzyme activity obtained for amino acid substitutions at Glu-461 and Tyr-503.

Histidine 464: Since the His-464 mutants give comparable results to Met-3 mutants, it seems probable that enzyme activity is reflecting suppressor efficiency at this site also. This lack of a unique suppression pattern, combined with the fact that all of the amino acid substitutions at His-464 result in a Lac^+ phenotype, indicates that this residue is probably not part of the active site.

Glutamate 461: The only amino acid substitution at Glu-461 which results in an enzyme with more than 5% of wild-type activity is histidine. All of the other substitutions, whether introduced by missense mutation or nonsense suppression, result in enzyme activities of less than 1%. None of the enzymes, including the histidine mutants, has sufficient biological activity to allow the cells to use lactose as a carbon source. This data supports previous biochemical evidence that Glu-461 is involved in catalysis. Preliminary kinetic data on the mutant proteins provides confirmation (R. E. HUBER and C. G. CUPPLES, unpublished data). It is interesting that the histidine substitution gives the highest activity of any of the mutant proteins. In the currently accepted model for β-galactosidase catalysis, the glutamate residue provides a negative charge to stabilize the positively charged, galactosyl-enzyme intermediate (reviewed by SINNOTT 1987). Clearly, although histidine can function as a proton acceptor, it cannot provide a negative charge *per se*. An alternate possibility is the formation of a covalent bond between the enzyme and the intermediate, a possibility that has been discussed previously (SINNOTT

1978). Kinetic studies currently in progress should clarify this point.

Aspartate is not a good substitute for glutamate despite its negative charge, perhaps because the side group does not extend far enough into the active site. Aspartate was found to be a poor substitute for the glutamate residue in the active site of triosphosphate isomerase, also (STRAUS *et al.* 1985).

Tyrosine 503: The 7 amino acid substitutions produced by introducing missense mutations at Tyr-503 reduce the number of units of enzyme activity to below 0.1%. The substitutions produced by nonsense suppression show a similar reduction in enzyme activity. The dramatically decreased activity of the mutant proteins strongly supports previous, indirect evidence that Tyr-503 is the active site tyrosine (FOWLER *et al.* 1978; SINNOTT and SMITH 1978).

Phenylalanine and histidine are the best replacements for tyrosine in the collection of mutant enzymes produced by missense mutation. Phenylalanine is identical to tyrosine with the exception of the hydroxyl group on the tyrosine phenolic ring. Since it is this group which would provide the proton for catalysis, it is unlikely that phenylalanine has any catalytic activity *per se*. However, if the phenylalanine causes minimal disruption of the active site, the substrate will still be able to bind and the Glu-461 carboxylate group can still function effectively to break the glycosyl bond. The absence of an acid catalyst simply reduces the rate of the reaction. Either the size or the charge of the histidine, or both, may make it one of the better substitutions for tyrosine.

Cysteine and lysine substitutions produced by nonsense suppression also produce relatively high levels of enzyme activity. However, the corresponding missense mutants have very low activities. Other workers have found levels of activity for lysine 503 and cysteine 503 mutants which are comparable to our missense mutants (M. RING and R. E. HUBER, personal communication), indicating that the levels produced by the missense mutants are the correct ones. We are unable to account for the discrepancy between the activities of the suppressed nonsense and the missense mutants. One possibility is mischarging of the suppressors with tyrosine, at such a low level (less than 1%) that we are unable to detect it by protein sequencing. Whatever the explanation, the relative differences in activity between the suppressed nonsense mutations and the missense mutations are minor in comparison with the very large decrease in activity of the mutant proteins compared with the wild type.

Possible alteration of substrate specificity: Levels of more than 100 units of wild-type β-galactosidase activity correlate with a Lac^+ phenotype (SILHAVY, BERMAN and ENQUIST, 1984; SIMONS, HOUMAN and KLECKNER 1987; our unpublished data). However, the His-461 missense mutant produces almost 300 units of β-galactosidase and is Lac^-. Either ONPG is a better substrate for the mutant protein than it is for the wild type, or lactose is a poorer substrate for the mutant protein than for the wild type.

The relative intensity of the blue color produced by many of the mutants on Xgal plates does not correlate with the levels of β-galactosidase activity measured with ONPG. For example, the Ala-461 missense mutant produces 28 units of activity compared with the 1 unit produced by Asp-461 mutant but the latter is blue and the former remains white even after prolonged incubation. This is in contrast to our experience with *lacZ* mutations outside the active site; for these, we have consistently found that the intensity of the blue color on Xgal plates is an accurate reflection of the units of β-galactosidase measured with ONPG (J. H. MILLER, unpublished results). The unusual behavior of the active site mutants probably reflects variations in the efficiency of interaction between the mutant enzymes and the two substrates, Xgal and ONPG. The relative levels of activity of two mutant enzymes may not remain the same from substrate to substrate.

In contrast to the Glu-461 mutants, all of the cells containing Tyr-503 missense mutants produce blue colonies even when the number of units of β-galactosidase activity is four to five orders of magnitude below that of the wild type. This may indicate that ONPG is a poor substrate, compared to Xgal, for all of the Tyr-503 mutant enzymes. Alternatively, it may be that measuring the hydrolysis of ONPG *in vitro* is a less sensitive assay for low levels of enzyme activity than detecting the hydrolysis of Xgal *in vivo*.

Conclusions: The data presented in this paper clearly show that most, if not all, amino acid substitutions at either Glu-461 or Tyr-503 result in greatly decreased levels of enzyme activity. While we cannot rule out the possibility that the replacements are producing subtle structural changes in the mutant enzymes which interfere with activity indirectly, the results do agree with previous biochemical data on the roles of these two residues in catalysis. Kinetic analysis of the mutant proteins should provide confirmation.

The suppressor screening system has proven to be very useful for rapidly producing and screening multiple β-galactosidase mutants. The major advantages of the system are particularly well illustrated in the case of the histidine substitution at Glu-461. There was no *a priori* reason to construct a histidine missense mutant since the current schemes for β-galactosidase catalysis require a negatively charged amino acid at this site. The suppressor screening system allowed us to quickly and easily test the effects of a substitution which we would otherwise not have tried. Further

analysis of the histidine mutant may help to clarify the role of Glu-461 in catalysis.

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LITERATURE CITED

BOEKE, T. D., 1981 One and two codon insertion mutants of bacteriophage f1. Mol. Gen. Genet. **181:** 288–291.

BOSSI, L., 1983 Context effects: translation of UAG codon by suppressor tRNA is affected by the sequence following UAG in the message. J. Mol. Biol. **164:** 73–87.

BRAKE, A. J., A. V. FOWLER, I. ZABIN, J. KANIA and B. MÜLLER-HILL, 1978 β-Galactosidase chimeras: primary structure of a *lac* repressor-β-galactosidase protein. Proc. Natl. Acad. Sci. USA **75:** 4824–4827.

DAVIS, L. G., M. D. DIBNER and J. F. BATTY, 1986 *Basic Methods in Molecular Biology.* Elsevier, New York.

FOWLER, A. V., I. ZABIN, M. L. SINNOTT and P. J. SMITH, 1978 Methionine 500, the site of covalent attachment of an active site-directed reagent of β-galactosidase. J. Biol. Chem. **253:** 5283–5285.

GALAS, D. J., M. P. CALOS and J. H. MILLER, 1980 Sequence analysis of Tn9 insertions in the *lacZ* gene. J. Mol. Biol. **144:** 19–41.

HERRCHEN, M., and G. LEGLER, 1984 Identification of an essential carboxylate group at the active site of *lacZ* β-galactosidase from *Escherichia coli.* Eur. J. Biochem. **138:** 527–531.

LANGRIDGE, J., 1974 Mutation spectra and the neutrality of mutations. Aust. J. Biol. Sci. **27:** 309–320.

LANGRIDGE, J., and J. H. CAMPBELL, 1969 Classification and intragenic position of mutations in the β-galactosidase gene of *Escherichia coli.* Mol. Gen. Genet. **103:** 339–347.

LEGLER, G., and M. HERRCHEN, 1983 N-Substituted D-galactosylamines as probes for the active site of β-galactosidase from *Escherichia coli.* Carbohydr. Res. **116:** 95–103.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MILLER, J. H., 1972 *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MILLER, J. H., and A. M. ALBERTINI, 1983 Effects of surrounding sequence on the suppression of nonsense codons. J. Mol. Biol. **164:** 59–71.

MILLER, J. H., and W. S. REZNIKOFF, 1978 *The Operon.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MILLER, J. H., C. COULONDRE, M. HOFER, U. SCHMEISSNER, H. SOMER, A. SCHMITZ and P. LU, 1979 Genetics studies of the *lac* repressor IX. Generation of altered proteins by the suppression of nonsense mutations. J. Mol. Biol. **131:** 191–222.

MÜLLER-HILL, B., and J. KANIA, 1974 *Lac* repressor can be fused to β-galactosidase. Nature **249:** 561–563.

NORMANLY, J., J.-M. MASON, L. G. KLEINA, J. ABELSON and J. H. MILLER, 1986 Construction of two *Escherichia coli* amber suppressor genes: $tRNA^{Phe}_{CUA}$ and $tRNA^{Cys}_{CUA}$. Proc. Natl. Acad. Sci. USA **83:** 6548–6552.

SILHAVY, T. J., M. L. BERMAN and L. W. ENQUIST, 1984 *Experiments with Gene Fusions.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SIMONS, R. W., F. HOUMAN and N. KLECKNER, 1987 Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:** 85–96.

SINNOTT, M. L., 1978 Ions, ion-pairs, and catalysis by the *lacZ* β-galactosidase of *Escherichia coli.* FEBS Lett. **94:** 1–9.

SINNOTT, M. L., and P. J. SMITH, 1978 Affinity labelling with a deaminatively generated carbonium ion. Biochem. J. **175:** 528–538.

SMITH, K. A., S. F. KNOWLAN, S. A. MIDDLETON, C. O'DONOVAN and E. A. KANTROWITZ, 1986 Involvement of tryptophan 209 in the allosteric interactions of *Escherichia coli* aspartate transcarbamylase using single amino acid substitution mutants. J. Mol. Biol. **189:** 227–238.

STRAUS, D., R. RAINES, E. KAWASHIMA, J. R. KNOWLES and W. GILBERT, 1985 Active site of triosephosphate isomerase: *In vitro* mutagenesis and characterization of an altered enzyme. Proc. Natl. Acad. Sci. **82:** 2272–2276.

WELPLY, J. K., A. V. FOWLER and I. ZABIN, 1981 β-Galactosidase α-complementation: effect of single amino acid substitutions. J. Biol. Chem. **256:** 6811–6816.

ZABIN, I., 1982 β-Galactosidase α-complementation. A model for protein-protein interaction. Mol. Cell. Biochem. **49:** 87–96.

ZABIN, I., and A. V. FOWLER, 1978 β-Galactosidase, the lactose permease protein, and thiogalactoside transacetylase. pp. 89–121. In: *The Operon,* Edited by J. H. MILLER and W. S. REZNIKOFF. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

ZOLLER, M. J., and M. SMITH, 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. **10:** 6487-6500.

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