GENETIC EVIDENCE FOR THE DISPOSITION OF THE SUBSTRATE BINDING SITE OF β-GALACTOSIDASE

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Communicated by S. Spiegelman, May 10, 1968

It is generally believed that an enzyme's characteristic properties of specificity and catalytic ability are localized in a particular region or in regions of the enzyme that are much smaller than the whole enzyme molecule. There are, however, indications that this "active site" may not correspond to a single, sharply defined region of the protein, but rather may be disposed in a non-contiguous fashion along the polypeptide chain, which is folded to bring the various parts of the active site into proximity with the substrate. In particular, the X-ray analysis of lysozyme in the presence of a substrate analogue[1] directly demonstrates that amino acid side chains distant from one another participate in substrate binding.

The question of the disposition of substrate binding sites within an enzyme may possibly be approached genetically by determining the map position of mutations that inactivate the enzyme by reducing its affinity for substrate. Consequently, studies have been made of a large number of mutant forms of β-galactosidase of *Escherichia coli* with the aim of obtaining mutations that affect substrate affinity. All mutations have been approximately mapped to determine whether the substrate binding site is in a localized region or distributed along the polypeptide chain. This paper reports the isolation, characterization, and mapping of 16 mutations that affect the substrate binding properties of β-galactosidase. A summary of these and other data relating to organization within the gene for β-galactosidase will appear elsewhere.[2]

Material and Methods.—Bacterial strains: The experimental organism was *E. coli* K-12. Many of the β-galactosidase mutants used in these investigations were from the collections of the Service de Biochimie Cellulaire et de Génétique Microbienne of the Institut Pasteur; 470 others were induced in the Hfr (Hayes) stock 3300 ($i^- o^+ z^+ y^+$).

Media: Minimal and complete liquid and agar media were as described by Pardee *et al.*;[3] lactose tetrazolium agar was prepared by the method of Zamenhof.[4]

Mutagenesis: Mutations were induced by means of *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (K and K Laboratories). Log phase cells were suspended in 0.5 ml of 0.2 *M* acetate buffer (pH 5.0) containing 400 μg of the mutagen and were left at 37°C for 2 hr. The cells were then washed, resuspended in complete medium, frozen by immersion in liquid air, and stored at −16°C until required. To isolate mutants, the mutated stock was diluted and plated on lactose tetrazolium agar, where mutants unable to utilize lactose appear as red colonies or sectors.

Classification of mutants: Mutant colonies were purified and tested for growth on lactose and glucose; those growing on glucose but not on lactose were kept for further study. To assay β-galactosidase qualitatively, colonies on glucose plates were treated with toluene and ortho-nitrophenyl-β-galactoside (ONPG).[5] The ONPG (Light and Co.) was recrystallized from ethanol before use. Mutants with little or no enzyme were tested for suppression by two amber suppressors (su_I^+ and su_{II}^+) and two ochre suppressors (su_B^+ and su_C^+) by the method of Newton *et al.*[6] In mutants that were nonsuppressible, cross-reacting material (CRM) was assayed by the competition method of Yanofsky and Stadler.[7]

Preparation of enzyme extracts: Mutant strains were grown overnight with shaking in complete medium at 37°C, centrifuged, suspended in phosphate buffer at pH 7 without β-mercaptoethanol, disrupted by sonic oscillation, and recentrifuged. Inducible mutants were grown with the addition of 10^{-3} *M* isopropyl-β-thiogalactoside as inducer. Partially purified extracts were prepared by adding streptomycin to a concentration of 2.5%, centrifuging off the precipitated nucleic acids, and precipitating the protein fraction containing β-galactosidase by the addition of saturated ammonium sulfate to 64% of saturation. Protein was estimated by means of the Folin-Ciocalteau reagent.[8]

Preparation of galactosides: The substrate, para-nitrophenyl-β-D-galactopyranoside, and the inhibitors, ortho- and para-nitrothiophenyl-β-D-galactopyranoside, were synthesized from tetraacetyl-galactosyl-bromide and the appropriate substituted phenol by the Koenigs-Knorr reaction. The uncorrected melting points were as follows: para-substrate 172°, ortho-inhibitor 186°, para-inhibitor 170°C.

Kinetics: Initial velocities were calculated from the increase with time of optical density at 405 mμ in a Zeiss PMQII spectrophotometer. The reciprocals of the initial velocities were plotted against the reciprocals of substrate concentration to provide Michaelis constants (K_m) and maximum velocities (V). Competitive inhibition constants (K_i) were determined graphically.[9] Confidence limits for kinetic constants were obtained by the weighting and computational procedures of Wilkinson.[10] All reactions were carried out in 0.1 *M* sodium phosphate buffer (pH 7.0) containing Mg and Mn ions and β-mercaptoethanol,[11] usually at room temperature. Under these conditions, 1 mg of unfractionated protein from wild-type bacteria hydrolyzed 84 μmoles of ONPG per min. Most mutants possessed less than 1% of this activity.

Mapping: The approximate positions of mutations of the β-galactosidase gene were determined by sexduction with episomally substituted deletions that divided the gene into 25 map regions. Most deletions were of the overlapping type;[12] other deletions[13] were transposed from F^- strains to episomes.

Crosses were made on agar plates containing mineral salts, thiamin, and lactose by the superimposition of drops from cell suspensions of the mutants and deletions; mutants outside the region of the deletion usually gave 100–300 lactose-positive recombinants per cross.

Results.—Kinetic characteristics of β-galactosidase: Mutant enzymes were tested for altered binding in terms of the ability of lactose to inhibit competitively the hydrolysis of ONPG. This method requires that a two-substrate system follow competitive inhibition kinetics and that K_m represent the equilibrium constant for the dissociation of substrate from enzyme. The expected kinetics were observed both in a double reciprocal plot and in a reciprocal plot of fractional inhibition against inhibitor concentration. In the latter plot, the curve intercepted the ordinate at unity, indicating that lactose inhibition of ONPG is completely competitive and that lactose is not appreciably bound at any site unavailable to ONPG. The identity of the ONPG and lactose sites is important because, as examples below indicate, the binding of lactose may be eliminated by mutation without gross change in the binding of ONPG.

The nature of K_m was determined by demonstrating the identity of the K_m for para-nitrophenyl-β-galactoside (3.6×10^{-5} *M*) with the K_i for para-nitrothiophenyl-β-galactoside (3.3×10^{-5} *M*). These compounds are structurally the same except for the replacement of the oxygen atom of the glycosidic linkage with a sulfur atom. Since the thiogalactoside is not hydrolyzed, K_i for this compound is the dissociation constant for the reaction β-galactosidase + thiogalactoside $\rightleftharpoons$ β-galactosidase-thiogalactoside complex. The K_m for the *O*-galactoside is expected to be identical with the K_i for the *S*-galactoside if the atoms

of the glycosidic linkage are not involved with binding to the enzyme and if K_m is the dissociation constant of the enzyme-substrate complex. The ortho-substituted nitrophenyl-*O*-galactoside gave a K_m of 2.9×10^{-4} M, which was nearly three times larger than the K_i for the sulfur analogue (1.0×10^{-4} M) probably because the ortho-substituted *O*-glycosides exhibit hindrance of free rotation of the benzene ring, whereas the corresponding *S*-glycosides do not.[14]

In the above comparisons of K_m and K_i, the difference for the ortho-compounds was statistically significant, but that for the para-compounds was not. Values for K_m and V have been calculated from a weighted regression of $1/v$ against $1/S$. The confidence limits of the intercepts for K_m and V were found to vary little for different determinations, so coefficients of variation were calculated for these constants measured under standard assay conditions. The coefficient of variation for K_m was 3.3 per cent; for V, it was 10.8 per cent.

To test mutant enzyme preparations for changes in lactose binding, the reciprocal velocity for ONPG hydrolysis was plotted against increasing lactose concentration. Mutant enzymes with normal binding give a slope extrapolating to a common intercept on the abscissa at $-K_i\,[1 + (S/K_m)]$, whereas enzymes with decreased binding have their intercepts displaced to an extent dependent upon the change in K_m or K_i.

Mutant enzymes with decreased lactose binding: The first mutant with β-galactosidase having a low affinity for lactose was isolated by Perrin.[15] This is mutant 13PO, which has a greatly reduced ability to bind both ONPG and lactose. To obtain further mutants of this type, enzyme extracts were prepared from mutants that were ambers or ochres, serine-suppressed ambers, mutants possessing immunologically cross-reacting protein, and mutants that could not be placed in any of these classes. Several mutants from each map region were tested for mutational changes in all parts of the β-galactosidase molecule. Accordingly, the altered β-galactosidase in 144 mutants was examined by the method outlined above for deviations from normal inhibition by lactose. The various types of mutants tested and their map positions are shown in Figure 1. This procedure yielded only nine mutants (6%) with decreased lactose binding.

When these mutants were examined more closely, it became apparent that many of them, although unable to grow on lactose, possessed significant enzyme activity when measured by ONPG hydrolysis. Therefore, an additional 120 lactose-negative mutants were tested for the ability to hydrolyze ONPG. Of these, 18 mutants had slight hydrolytic activity toward ONPG, and 6 of these 18 had enzymes with decreased affinities for lactose. However, the total frequency of mutants with altered substrate affinity among all mutants of the β-galactosidase gene that prevent growth on lactose is only about 4 per cent. Among CRM-formers, including serine-suppressed ambers, the frequency is about 9 per cent. Only 8 mutants of the 150 tested had insufficient enzyme activity for kinetic analysis.

In Figure 1, the roman numerals indicate the map positions at which mutation leads to altered binding constants. Five apparent binding sites have been found, containing one mutant in site I, four in site II, three in site III, six in site IV, and two in site V.

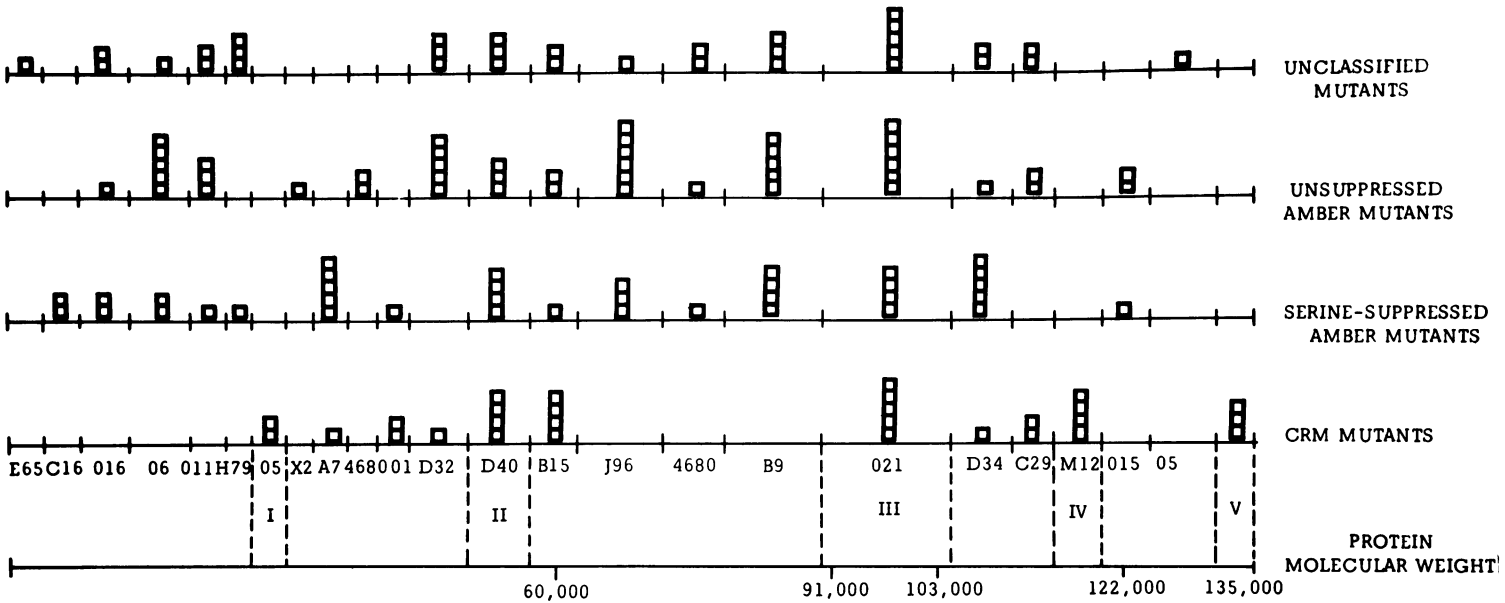

FIG. 1.—Classification and map distribution of mutants examined for changes in β-galactosidase specificity. The letters and figures in the second lowest line identify the episomal deletions used in mapping. The lowest line gives the molecular weights for peptides produced by amber mutations at the positions shown[17] and thus serves to orient the deletion map. Numbers of mutant enzymes examined for each deletion region are shown in the form of columns. The roman numerals indicate the map regions in which mutation may alter the substrate affinity of β-galactosidase.

Binding constants of mutant enzymes: Table 1 lists Michaelis constants and maximum velocities for ONPG given by wild-type β-galactosidase and the enzyme from mutants with altered binding. Also shown are the competitive inhibition constants for lactose and galactose.

Generally the binding of all three compounds is decreased in the mutants; the lower the affinity of a compound for the enzyme, the greater the decrease in affinity in the mutant. Since galactose has the least affinity for the substrate binding site, its binding usually is most affected. However, there is no clear proportionality in the effect of a given mutation on the binding of ONPG, lactose, and galactose. Nor does there seem to be a change in substrate binding at a particular site that is characteristic of all mutants at that site and unique to it.

TABLE 1. *Kinetic constants of β-galactosidase from wild-type (3300) strain and from mutants with altered substrate affinities.*

Strain	Site	V for ONPG	K_m for ONPG	K_i for lactose	K_i for galactose
3300	—	107	0.26	1.5	7.5
13PO	I	0.03	5.9	158	7.5
U120	II	0.02	0.25	2.4	500
141	II	0.04	2.0	30	1900
132	II	0.5	0.67	50	710
U116	II	18.6	5.9	170	112
1260	III	0.02	0.33	12	78
Y17	III	0.5	0.6	>1000	1450
833	III	30.3	5.5	>1000	*
261	IV	17.5	0.61	34	375
875	IV	20.9	0.61	81	450
U144	IV	1.3	0.31	400	1300
2612	IV	0.03	0.58	>1000	*
2514	IV	2.2	0.6	>1000	*
990	IV	0.3	0.6	32	330
269	V	<0.01	0.62	42	250
2133	V	1.5	2.0	19	225

Michaelis constants (K_m) and inhibition constants (K_i) in mM concentration; maximum velocities (V) in μM ONPG hydrolyzed per min per mg of protein. Coefficients of variation: K_m and K_i, 3.3%; V, 10.8%.

* No detectable inhibition.

Although the enzymes from some mutants, such as 132 and 261 or Y17 and 2514, have generally similar relative affinities for the three substrates tested, these mutants occur at different sites. The enzymes of certain mutants, Y17, 2612, and 2514, have lost all measurable affinity for lactose and galactose while retaining most of their affinity for ONPG. However, as shown above, no evidence has yet been found for a second binding site on the β-galactosidase molecule.

Ordering of mutations within a site: Several of the binding-site regions include mutations with effects varying from relatively small changes in substrate binding to very large changes. Such a distribution within a site suggests that these mutations may not change the amino acids directly concerned with binding, but may merely indicate their general location. If this postulate is correct, the further away the amino acid change from the amino acids in direct contact with the substrate, the less should be its effect in altering binding.

The appropriate technique for determining mutation order is that of Jacob and Wollman,[16] in which the segregation of the regulator gene (*i*) is observed in lactose-positive recombinants resulting from crosses between i^+ and i^- mutants of the β-galactosidase gene. Mutants with enzymes of reduced substrate affinity and the required regulator gene constitution were available only for site III. The order of these mutations, together with the substrate affinities of the corresponding enzymes, is shown in Figure 2.

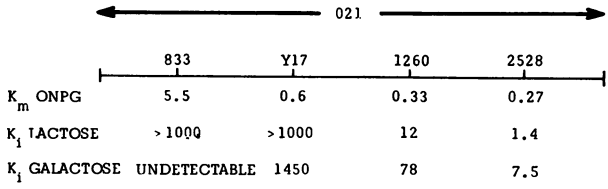

FIG. 2.—The order of mutations in deletion region 021 (substrate-binding site III) and the Michaelis and inhibition constants for their enzymes. Concentrations are in mM.

The data demonstrate that the loss of affinity in the enzymes of the mutants follows a gradient from mutant 2528, which is not significantly different from wild type, to mutant 833, which has lost the ability to bind both lactose and galactose. On the hypothesis that severity of binding change is proportional to the distance of the amino acid replacement from the contact amino acids, the latter are expected to be near the position in the enzyme where mutation 833 is expressed.

Discussion.—The mutants described above, with enzymes of altered substrate-binding properties, probably do not have changes in the amino acids in actual contact with the substrate. They are more likely to represent examples of amino acid replacements on the surface of the enzyme that alter the affinity between enzyme and substrate if they occur within a minimal distance of the contact amino acids. The mutational replacement of a contact amino acid may result in a completely inactive protein. Several mutants that map at sites II and III are quite devoid of enzyme activity, although they do make cross-reacting protein.

The mapping data demonstrate that mutations that change the binding properties of β-galactosidase are not randomly distributed along the gene, but are concentrated in specific regions. This finding may indicate that the amino acid residues in contact with the substrate are dispersed in five distinct groups that are brought into proximity with each other by the folding pattern of the polypeptide chain of the active enzyme. There are, however, three objections to the conclusion that the substrate binding region of β-galactosidase is composed of five groups of residues. Firstly, additional sites might be revealed if a larger sample of mutants were examined. Secondly, the mutant enzymes that have decreased substrate affinity also have decreased maximum velocities. The question then arises whether the binding-site mutants examined represent a specially selected class in which both binding and catalysis are simultaneously

decreased by a single mutation. This dual character of the mutants is partly due to the fact that a mere reduction in lactose binding, unless it is extreme, is not sufficient to result in mutants that are completely lactose-negative. Only mutants Y17, 833, 2612, and 2514, with enzymes in which lactose-binding is absent or negligible, are likely to be lactose-negative because of binding changes alone. Even these mutant enzymes have reduced maximum velocities for ONPG. The enzyme from mutant 833 is an exception. Its maximum velocity for ONPG is given as about 30 per cent of that of the wild-type enzyme (Table 1), but the isolated enzyme is very unstable. In buffer at room temperature, it loses activity at the rate of 15 per cent in 10 minutes, so its maximum velocity in the cell may approach that of the wild-type enzyme. An amino acid replacement need not necessarily affect both the Michaelis constant and the maximum velocity for a substrate because mutants have been found (Langridge, unpublished) that have enzymes with increased affinity but without marked change in maximum velocity. These mutants were obtained by selection for growth on β-galactosides with very low affinity for wild-type β-galactosidase. Although it has been shown that these mutations are located in the gene for β-galactosidase, no method has been found for determining their position within the gene. Even if the possibility of selection bias remains, the only error such bias is likely to introduce is that of underestimating the number of binding sites.

The third objection arises from the finding that two of the apparent substrate binding sites appear to be adjacent to, or include, regions of association among monomers to form the active tetrameric enzyme (Langridge, unpublished). Some of the enzymes altered at a particular binding site have both reduced affinity and loosened quaternary structure, whereas others at the same site have changes only in one characteristic. The bearing of these observations on the number of substrate binding sites is not known because the relation between substrate affinity and monomer association in β-galactosidase is at present obscure.

Summary.—Most mutants of the gene for β-galactosidase in *E. coli* have enzymes with normal substrate binding properties but greatly reduced maximum velocities. Only about 4 per cent of mutant enzymes have changes in substrate affinity. The Michaelis constants and maximum velocities for ONPG and the competitive inhibition constants for lactose and galactose have been measured for 16 mutants with decreased substrate binding. These mutations are located in five separate regions of the gene for β-galactosidase. Changes in substrate affinity appear to occur only if the mutation affects amino acids within a limited distance of a binding site. With certain qualifications, present evidence suggests that the binding region of the active enzyme is produced by folding of the polypeptide chain to bring at least five sequences of amino acids into juxtaposition about the substrate.

I am grateful to Dr. J. Monod for his hospitality at the Service de Biochimie Cellulaire of the Institut Pasteur. I wish also to thank Drs. J. Monod, F. Jacob, W. A. Newton, and M. Malamy for bacterial strains and generous advice.

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