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Genetic and Enzymatic Experiments relating to the Quaternary Structure of β-Galactosidase

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Abstract

The quaternary structure of β-galactosidase, which consists of four identical subunits, has been studied by the isolation and characterization of appropriate mutants of *Escherichia coli*. Of 146 mutants examined, 19 were found to have enzymes with reduced subunit association. These altered enzymes are especially sensitive to inactivation by urea which, at concentrations that do not affect the normal enzyme, causes dissociation into subunits. Mapping with overlapping deletions showed that the mutations affecting quaternary structure are not distributed continuously, but occur in five or six groups within the gene. The mapping indicates that polypeptide sequences involved in subunit association and in substrate binding are contiguous. A model for β-galactosidase structure is suggested in which substrate binding sites are provided by the clefts formed between subunits when they associate.

Introduction

There are great technical difficulties in attempting to study the structure of large proteins by means of amino acid sequence analysis and the determination of conformation by X-ray diffraction. Therefore, attempts have been made to use a combined genetical–biochemical approach to obtain information on the gross architecture of β-galactosidase (EC 3.2.1.23) and its relation to the enzyme's function. Previous studies were concerned with the distribution of substrate-binding sites within the enzyme (Langridge 1968*a*) and with the pattern of tertiary structure (Langridge 1968*b*). In the course of these experiments it was found that some mutations appeared to inactivate the enzyme by causing its dissociation into subunits.

The β-galactosidase of *Escherichia coli* in its catalytically active state has a molecular weight of 518 000 (Sund and Weber 1963) and is composed of four identical subunits with particle molecular weights of 125 000–135 000 (Zipser 1963). Judging from the fact that dissociation is brought about by such substances as guanidine, urea and dodecyl sulphate (Wallenfels *et al.* 1963), the subunits are held together by hydrophobic interactions and perhaps hydrogen bonds. A relatively small part of the polypeptide chain would be needed to establish such interactions, which implies the presence of localized sequences of amino acids primarily concerned with the association of the subunits to form the quaternary structure of the enzyme. Since there is a linear relationship between the sequences of deoxyribonucleotides and amino acids (Fowler and Zabin 1966) there may be a localized distribution of mutations that affect this quaternary structure. The experiments reported here attempt to test this proposition.

Materials and Methods

Experiments were made on *E. coli* K-12 Hfr Hayes and various mutants of this strain.

In earlier experiments (Langridge and Campbell 1969) 174 independent mutations of the β-galactosidase gene were isolated following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The enzymes of most of these mutants, together with others provided by Drs J. Monod, F. Jacob and P. Bergquist, were tested for alterations in subunit association. Tests were made of 146 mutant enzymes, usually by measuring their sensitivity to urea (1·0M for 2 h at 37°C) and sometimes by testing their sensitivity to hydrogen ions (pH 5·5). After incubation in urea the enzymes were diluted 10-fold with phosphate buffer for assay. During β-galactosidase assay the diluted enzymes were in contact with concentrations of urea varying from 0·05 to 0·5M according to the experiment, but for too short a period (about 15 min) for such low concentrations to affect the enzymes. The urea was always recrystallized from aqueous ethanol just before use to remove isocyanate, which reacts with the terminal (and epsilon) amino groups of proteins.

β-Galactosidase from wild-type (3300) and mutant strains (833 and Y17) was prepared and purified as follows. Twelve-litre cultures of each strain were grown overnight in yeast extract and peptone medium at 37°C. Bacterial cells were isolated by centrifugation, suspended in 50 ml of 0·1M phosphate buffer containing β-mercaptoethanol (0·1M) and broken by sonication. The cell-free extract was treated with streptomycin sulphate (2·5%) to remove nucleic acids and the protein precipitated at a concentration of 64% $(NH_4)_2SO_4$. The precipitate was redissolved in buffer and brought to a concentration of 30% $(NH_4)_2SO_4$. The resulting precipitate was dissolved in 15 ml of buffer and further purified by starch block electrophoresis. Separation on starch was carried out at 4°C for 16 h at a current of 50 mA in a buffer of pH 7·0 composed of sodium phosphate and sodium citrate, each at 0·2M. Of the two electrophoretic bands possessing β-galactosidase activity that were obtained, the more active one was eluted and used in later experiments. About 0·5 g of purified β-galactosidase was usually obtained.

Chromatography of purified β-galactosidase was performed with columns containing 0·5-mesh Agarose (Bio-Rad Laboratories, California, U.S.A.) in phosphate buffer containing β-mercaptoethanol. When subunit constitution was being examined the column was previously equilibrated with 4·5M urea in phosphate buffer. Fractions obtained from the column were assayed for β-galactosidase using *o*-nitrophenyl β-galactoside as substrate (Langridge 1968*a*). The fractions from urea-equilibrated columns were dialysed for 24 h against phosphate buffer before assay.

Results

Identification of Mutant Enzymes with Altered Quaternary Structure

When normal β-galactosidase is incubated with increasing concentrations of urea there is only a slight loss of enzymatic activity until a concentration of about 3·5M is reached. Thereafter, activity drops suddenly until nearly all is lost at about 4·0M urea (Fig. 1). If isocyanate-free urea is used it is known that this loss of activity is caused by a urea-induced dissociation of the β-galactosidase into its subunits (Shifrin and Steers 1967). As will be shown later, mutant enzymes with weakened subunit association readily lose enzymatic activity, even at low urea concentrations. Most routine testing for mutations affecting quaternary structure was therefore done by incubating the crude enzymes for 2 h at a urea concentration of 1·0M, a concentration with little effect on enzymes with normal subunit association.

Where screening tests identified a urea-sensitive enzyme, a quantitative measure of urea sensitivity was provided by the urea concentration for half-inactivation, obtained by titration of the enzyme between 0 and 4·5M urea. These tests resulted in the accumulation of 19 mutants with β-galactosidases of reduced subunit association (Table 1). There was a clear distinction between mutant enzymes with the same reaction to urea as the wild-type enzyme and those that were sensitive.

Table 1. List of mutants containing β-galactosidase with altered quaternary structure or reduced substrate binding

Competitive inhibition constants for lactose binding, the urea concentration for half-inactivation and the effect of pH of the reaction mixture are given. Mutations are arranged in their approximate order in the gene for β-galactosidase

Strain	K_i (M) for lactose	Urea concn (M) for half-inactivation	pH response
3300	$1{\cdot}5\times10^{-3}$	3·6	Normal
Z32	>1	1·6[A]	Acid-inhibited
13PO	$1{\cdot}6\times10^{-1}$	3·7	Normal
X34	$1{\cdot}6\times10^{-3}$	1·5	Alkali-inhibited
U116	$1{\cdot}7\times10^{-1}$	0·25	Normal
U120	$2{\cdot}4\times10^{-3}$	3·5	Alkali-inhibited
19 *sup D*	$1{\cdot}8\times10^{-3}$	0·5	Alkali-inhibited
U147	8×10^{-2}	0·2	Normal
U132	5×10^{-2}	0·6	Normal
U141	3×10^{-2}	0·4	Normal
2066	$4{\cdot}2\times10^{-2}$	0·8	Acid-inhibited
Y17	>1	0·8	Acid-inhibited
833	>1	2·2[A]	Acid-inhibited
2528 *sup D*	10^{-2}	1·1	Normal
1260 *sup D*	$1{\cdot}2\times10^{-2}$	1·2	Normal
990	$3{\cdot}2\times10^{-2}$	0·7	Acid-inhibited
U144	4×10^{-1}	1·1	Acid-inhibited
2514	>1	2·4[A]	Normal
875	$8{\cdot}1\times10^{-2}$	0·6	Acid-inhibited
261	$3{\cdot}4\times10^{-2}$	0·5	Acid-inhibited
2612	>1	1·8[A]	Acid-inhibited
2133	$1{\cdot}9\times10^{-2}$	0·35	Acid-inhibited
269	$4{\cdot}2\times10^{-2}$	3·5	Normal

[A] Enzymes activated by low concentrations of urea.

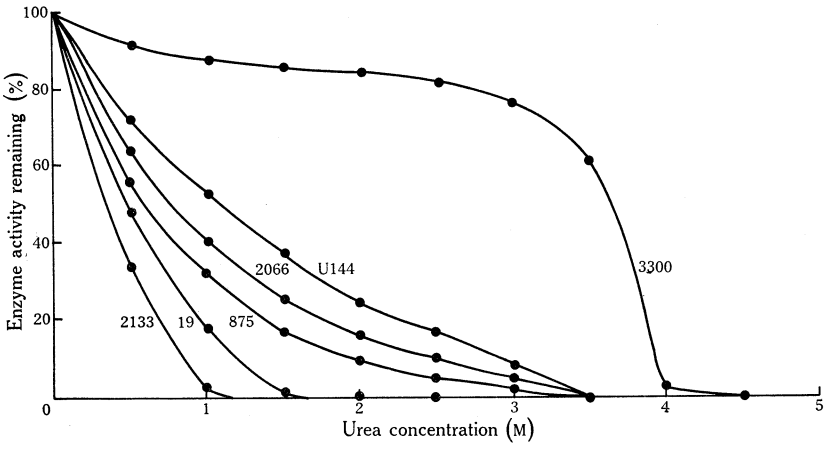

Fig. 1. Effect of urea concentration on the β-galactosidase activity of wild-type enzyme (3300) and the enzymes of a sample of mutants (U144, 2066, 875, 19, 2133). Enzyme extracts were incubated in urea for 2 h at room temperature and then diluted 10-fold in phosphate buffer for assay.

Effect of Urea

The urea inactivation of normal β-galactosidase is shown in Fig. 1 in comparison with several representative inactivation curves of mutant enzymes. The inactivation kinetics of the altered enzymes usually follow the same course, but, unlike the normal enzyme, there is no threshold urea concentration at which inactivation begins. Some altered enzymes (in mutants Z32, 833, 2514 and 2612 of Table 1) become more active catalytically at certain urea concentrations. Fig. 2 gives an example of such an activated enzyme, together with the usual inactivation curves for wild type and mutant. It was generally found that activation only occurred at relatively low urea concentrations, while further increases in concentration led to inactivation at the rate typical of mutant enzymes with reduced subunit association.

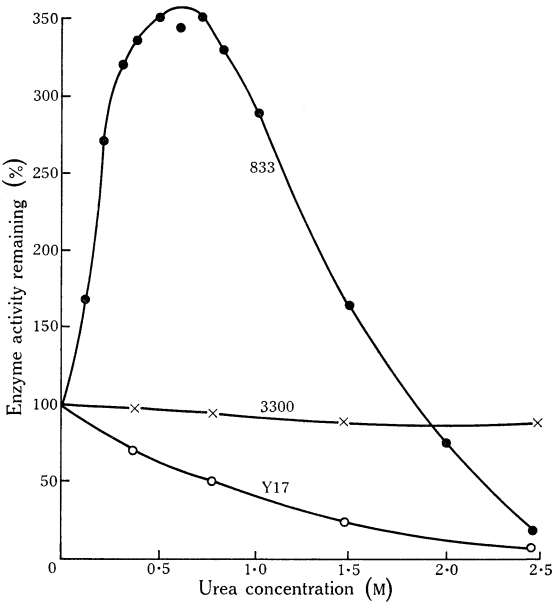

Fig. 2. Effect of urea concentration in activating hydrolysis by the altered β-galactosidase of mutant 833. Comparative urea–response curves are included for the enzyme of wild type (3300) and the usual type of mutant (Y17). The specific activities of the mutant enzymes as a percentage of that of 3300 were: 833, 30; Y17, 0·5.

In addition to estimates of urea sensitivity, Table 1 lists measurements of lactose binding in the form of competitive inhibition constants. It is evident that many mutant enzymes with altered quaternary structure also have reduced ability to bind lactose. However, there are exceptions. The enzymes of mutants X34 and 19 *sup D* (an amber mutant suppressed by the insertion of serine) are sensitive to urea but bind lactose normally, while the enzymes of mutants 13PO and 269 have reduced lactose affinity but are not sensitive to urea.

The possible relationship between a loosening of the subunit association of β-galactosidase and a decrease in the ability to bind substrate was examined by measuring the maximum velocity and substrate affinity of normal β-galactosidase preincubated for 2 h at room temperature with a range of urea concentrations. The results (Fig. 3) indicate that there is no change in substrate affinity even at urea concentrations that cause extensive subunit dissociation. Neither does the course of dissociation have any effect on tertiary structure as measured by heat sensitivity. With 4·5M urea, where the enzyme activity is reduced to 7% of its original value, the temperature sensitivity of

the remaining active enzyme is not significantly increased (half-life at 55°C: without urea, 91 min; with urea, 86 min). Moreover, two mutants known to form β-galactosidase mostly in the monomeric and dimeric form respectively were found to have substrate affinities identical to those of the normal enzyme [K_i (lactose) about 2×10^{-3} M], although they had greatly reduced maximum velocities. There appears, therefore, to be no necessary relationship between subunit dissociation and substrate binding.

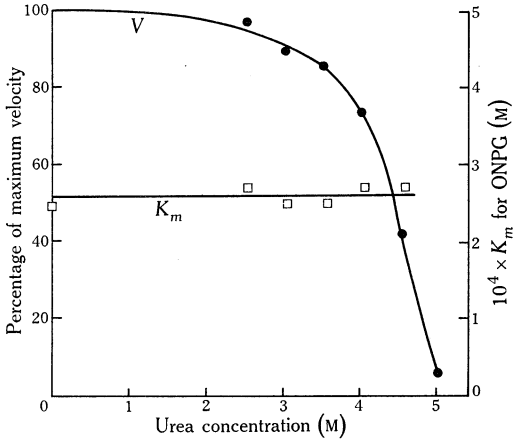

Fig. 3. Effect of urea concentration on the maximum velocity of hydrolysis (●) and binding (□) of *o*-nitrophenyl β-galactoside (ONPG) by the normal enzyme of strain 3300. Enzyme extracts were incubated in urea for 2 h at room temperature and then diluted 10-fold in phosphate buffer for assay.

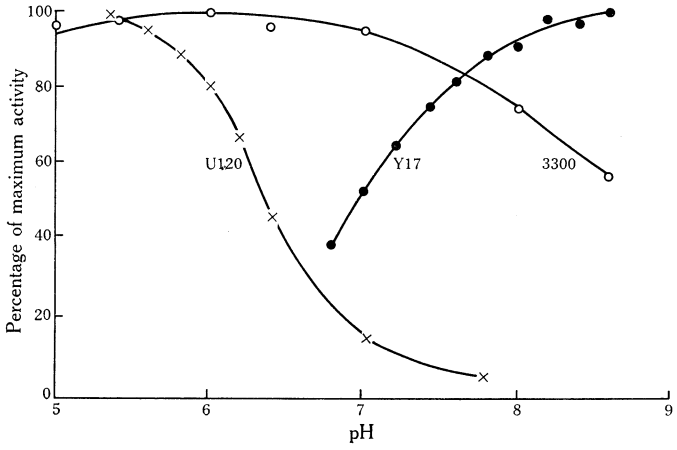

Fig. 4. Effect of pH of the reaction mixture on the β-galactosidase activity of wild type (3300) and of two differently sensitive mutants (U120 and Y17). Specific activities of the mutant enzymes at pH 7·0 as a percentage of that of 3300 were: U120, 0·02; Y17, 0·5.

Effect of Hydrogen Ion Concentration

Most of the mutants with altered quaternary structure are also sensitive to the pH of the medium in which catalytic activity is measured (Table 1). The majority are inhibited by acid pH, but a few that map towards the operator end of the gene (Fig. 7) are inhibited by alkaline pH. Fig. 4 gives representative pH–response curves for crude extracts of the two types of sensitive mutant and for the wild-type enzyme.

The hydrogen ion concentration could affect the ionization of an amino acid side-chain or it could reduce electrostatic interactions important in the association of subunits. To distinguish between these possibilities the β-galactosidase of one mutant (Y17), which is activated by alkaline pH, was purified by ammonium sulphate fractionation and chromatographed on a column of Sephadex G200 at pH 7·0 and 8·5 (Fig. 5). Fractions eluted at pH 8·5 were adjusted to pH 7·0 and left at room temperature for 2 h to allow the reassociation of any monomers present before enzyme assay. A good deal of enzyme inactivation occurred on the column, especially at pH 8·5, but as there was no protein at the expected position for monomers there was no evident change in the state of aggregation of subunits. The effect of pH on mutant enzyme activity therefore appears to be exerted through a change in protein conformation, perhaps by further altering the degree of subunit association, but not by changing an equilibrium between monomers and tetramers.

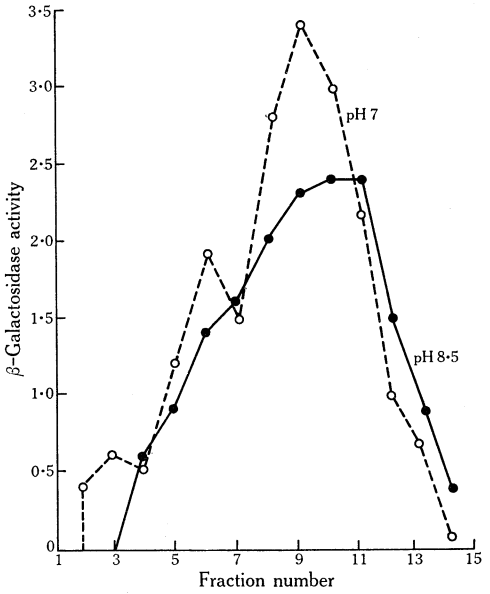

Fig. 5. Distribution of catalytic activity of the β-galactosidase of mutant Y17 after chromatography on Sephadex G200 at pH 7·0 and 8·5. Activity is expressed as $10^6 \times$ concn of *o*-nitrophenyl β-galactoside (M) hydrolysed per minute; fractions were numbered after the void volume (each fraction = 3 ml). Purified β-galactosidase (0·8 ml), which hydrolysed $4{\cdot}6 \times 10^{-5}$M *o*-nitrophenyl β-galactoside (10^{-3}M) per minute at pH 7·0 and 22°C, was added to a column with a total volume of 8·7 ml and a void volume of 40 ml.

Correlation between Urea Sensitivity and Subunit Association

Although urea is known to dissociate normal β-galactosidase into its component subunits, it is necessary to show that mutant enzymes have increased sensitivity to urea because their subunit interactions are much weaker. For this purpose the β-galactosidases of wild-type (3300) and mutant (833) bacteria were purified by ammonium sulphate fractionation and starch block electrophoresis as described in the Methods. The enzymes were then passed through a column of Agarose both in the absence and in the presence of 4·5M urea. Fractions from the columns not containing urea were assayed directly for β-galactosidase activity, while fractions from urea-equilibrated columns were dialysed to remove the urea and allow the subunits to reassociate before being assayed. The distribution of β-galactosidase activity for the two strains under both conditions is shown in Fig. 6.

The type of Agarose used in this experiment was chosen because it sieves molecules of from 10000 to 500000 molecular weight. Although tetrameric β-galactosidase of

molecular weight 518000 is almost completely excluded (Fig. 6), subunits of the enzyme, both dimers (molecular weight 250000; Steers and Shifrin 1967) and monomers (molecular weight 125000–135000; Zipser 1963), would be detected after restoration of the catalytic activity by reformation of the tetrameric condition. No monomeric subunits were found in solutions of normal or mutant β-galactosidase that were passed through Agarose previously equilibrated with phosphate buffer. When the Agarose column was equilibrated with 4·5M urea, apparently all of the β-galactosidase of mutant 833 dissociated on the column into monomeric subunits, but the normal enzyme was not in contact with urea long enough for there to be appreciable dissociation under these conditions (Fig. 6). This experiment confirms the assumption that β-galactosidases sensitive to urea have a weakened subunit association.

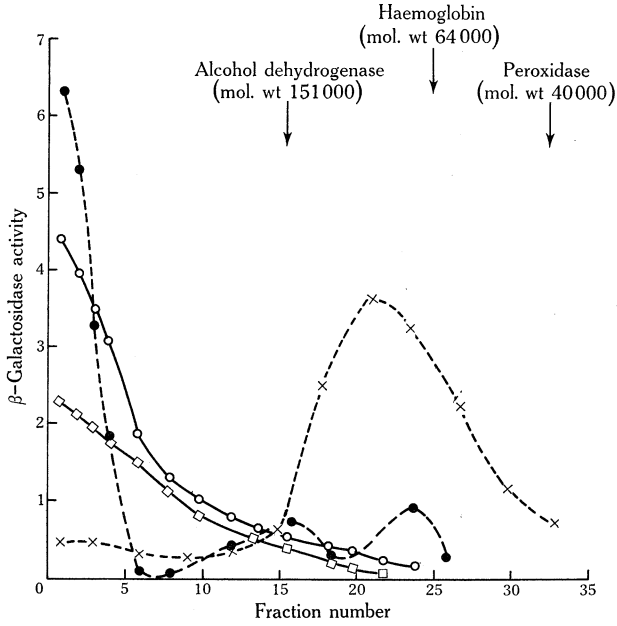

Fig. 6. Chromatography of β-galactosidase from wild type (3300) and mutant (833) on Agarose previously equilibrated with either phosphate buffer or urea. Purified enzyme (10 ml) dissolved in phosphate buffer was added to the column for each experiment (protein concentrations: strain 3300 enzyme, 18·9 mg/10 ml; strain 833 enzyme, 12·0 mg/10 ml). Activity is expressed as 10^5 × concn of *o*-nitrophenyl β-galactoside (M) hydrolysed per minute; fractions were numbered after the void volume (169 ml) as assessed by the appearance of blue dextran 2000.
Activity after elution with phosphate buffer: ○ 3300; □ 833.
Activity after elution in the presence of 4·5M urea and dialysis: ● 3300; × 833.

Intragenic Position of Mutations that affect Quaternary Structure

If the genetic alterations in quaternary structure are due to changes in amino acids that form part of the contact regions between subunits, their corresponding mutations are expected to map in discrete regions within the gene. If, however, generalized changes in the folded pattern of the polypeptide chain can secondarily reduce subunit association, no localization of the responsible mutations would be expected. To distinguish between the two possibilities the location of the mutations affecting quaternary structure was determined by mapping procedures. The position of many

of the mutations had been established previously (Langridge and Campbell 1969). Additional mutations were mapped by the same method of using overlapping and internal deletions.

Fig. 7 shows the position within the β-galactosidase gene of the 146 mutations whose corresponding enzymes were examined for their state of subunit association. The 19 mutations giving enzymes with reduced subunit association are shaded in the diagram. These mutations occur in five or possibly six groups closely corresponding to the regions previously found to contain mutations giving enzymes with low affinity for lactose and *o*-nitrophenyl β-galactoside (Langridge 1968*a*). In fact, the majority of mutant enzymes with poor substrate binding also have reduced subunit association.

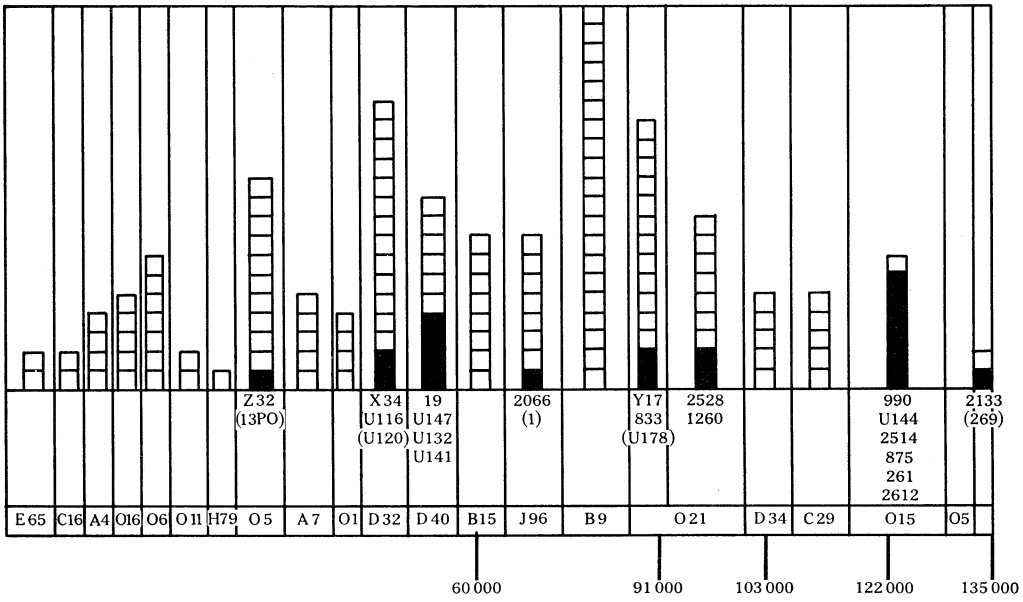

Fig. 7. Intragenic distribution of mutations affecting enzymes that were examined for changes in quaternary structure. The episomal deletions used in mapping are designated by the letters and numbers of the second lowest line. The lowest line gives the molecular weights of peptides produced by amber mutations at the positions indicated (Fowler and Zabin 1966) and thus orients the deletion map. Numbers of mutant enzymes examined for each deletion region are shown as columns. Mutants with altered quaternary structure are shaded in the columns and are identified by mutant number below the columns. The mutants enclosed in parentheses are mentioned in the text, but they do not have altered quaternary structure.

Discussion

The mapping data (Fig. 7) show a discontinuous distribution of mutations that reduce the association between subunits of β-galactosidase. This is in accord with the expectation that only certain specialized regions of the protein, probably comprising mainly hydrophobic amino acids, are concerned in subunit interaction.

Further evidence that these gene regions do correspond to nucleotide sequences coding for amino acids concerned with subunit association is provided by mutants 1 and U178. These two mutants were from the collection of Drs J. Monod and F. Jacob of the Institut Pasteur. The enzyme of mutant 1 was examined by Steers and

Shifrin (1967) and shown to be a dimer, while that of U178 has the molecular weight of a monomer (Perrin 1963). These two mutations have been mapped and their positions within the β-galactosidase gene are shown in Fig. 7. Both of them are located in regions identified as ones concerned with subunit associations.

A result of weakened quaternary structure is that the activities of the enzymes are now very sensitive to certain components of the reaction medium. They are particularly affected by hydrogen ion concentration, by bond-breaking reagents like urea and guanidine, by antibody to normal β-galactosidase and by substrate concentration. Such characteristics raise the possibility that enzymes whose activity is regulated by substrates and end-products may evolve by stabilization, through mutation, of the increased sensitivity produced by an earlier mutation that weakened the subunit association.

There are two possible explanations of the fact that most mutant β-galactosidases with altered quaternary structure also have a lower affinity for substrates. Either the weakening of the association between subunits causes a change in conformation affecting the substrate-binding sites, or the polypeptide sequences responsible for protein interaction and for substrate binding are contiguous or overlapping. Against the former possibility are the facts that there are mutant enzymes with only one of the two characteristics altered, that dissociation of the normal enzyme does not cause a change in substrate affinity or apparently alter tertiary conformation, and that the few active enzyme molecules of mutant enzymes composed mainly of dimers or monomers have normal substrate binding. The available evidence seems best interpreted as indicating that the association of protein subunits provides four clefts at the points of juncture in which substrate molecules are bound. This model would mean that sites for subunit association and substrate binding would be adjacent to one another and also that most mutations reducing the degree of association would have a secondary effect on the binding of substrate.

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