Evidence for Multiple Molecular Forms of Yeast β -Glucosidase in a Hybrid Yeast¹

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ABSTRACT

A mixture of β -glucosidases from *Saccharomyces fragilis* (Y-18) and *S. dobzhanskii* (Y-19) eluted from diethylaminoethyl cellulose in two peaks, whereas the enzyme from a hybrid, *S. fragilis* $\times$ *S. dobzhanskii* (Y-42), eluted in a single broad peak. The highest Y-42 activity fractions eluted at a sodium chloride molarity which was intermediate to the molarities at which most of the Y-18 and Y-19 activity was eluted. In cellulose polyacetate strips, Y-42 enzyme migrated as a diffuse band which spanned the distances migrated by the enzymes from the parent yeast strains. Antisera against either Y-18 or Y-19 enzyme precipitated 80 to 90% of Y-42 enzyme activity. When Y-42 enzyme was dissociated by heat or urea and reacted with parental antiserum, a concomitant increase in the opposite parental activity was demonstrable in both precipitation and complement-fixation (CF) tests. Urea-dissociated β -glucosidases were resolvable by sucrose-gradient centrifugation into multiple bands displaying specific CF activity. When the enzymes were exposed to 4 M urea for 12 min, particles of approximately 110,000 molecular weight were obtained. By extending the exposure time to 40 min, and incorporating 0.5 M urea in the gradients, smaller particles were detected with molecular weights ranging from 18,000 to 23,000. Attempts to regenerate enzyme activity after dissociation with urea were only moderately successful. Results suggested that a slightly acidic environment favored reassociation, as did the presence of 2-mercaptoethanol. Residual urea also seemed important. It is proposed that the structural genes for both Y-18 and Y-19 enzyme are present in Y-42 cells with either independent or closely interacting regulatory mechanisms. Since synthesis of the two parental-type polypeptides may be unequal, the availability of enzyme subunits for subsequent polymerization in the cell cytoplasm might be equalized at the polysome level. Random association of subunits would produce a binomial distribution of true hybrid enzyme molecules.

In the preceding report (11), we described procedures used for partially purifying the constitutive β -glucosidases from three strains of *Saccharomyces* yeasts. Physical and chemical characteristics of these enzymes were also presented. In the course of our work, the enzyme produced by a hybrid, *S. fragilis* $\times$ *S. dobzhanskii* (Y-42), displayed unique characteristics. The optimal pH

for hydrolysis of substrate by Y-42 enzyme fell between values for the enzymes from the parental yeast strains, *S. fragilis* (Y-18) and *S. dobzhanskii* (Y-19). By comparing affinity constants of certain β -glucosides for the enzymes, it also appeared that some values for the Y-42 enzyme were intermediate to those for the Y-18 and Y-19 enzymes. Initial urea-inactivation slopes for Y-42 enzyme and a mixture of Y-18 and Y-19 enzymes were superimposable. Moreover, Lineweaver-Burk plots and heat-inactivation slopes for the Y-42 enzyme were biphasic, suggesting at least a bimolecular system.

Although we originally felt that Y-42 preparations contained two molecular species of β -glucosidase which were characteristic of the enzymes synthesized by the parental yeast strains (8), addi-

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tional studies suggested that the hydrolyzing activity for β -glucosides in the hybrid yeast might actually be due to a broad spectrum of hybrid enzyme molecules composed of varying amounts of two distinct polypeptides. The purpose of this report is to present further evidence for the multiple molecular nature of β -glucosidase activity in the Y-42 yeast.

MATERIALS AND METHODS

Details concerning the yeast strains, growth media, culturing procedures, and techniques used for enzyme extraction and purification have been reported (11).

Antisera. Antibodies against partially purified preparations of Y-18 and Y-19 enzymes were produced in young, domestic rabbits. The animals received a series of five or six intramuscular injections at 6- or 7-day intervals. Enzyme preparations of approximately 10,000 units per ml were first emulsified with equal volumes of complete Freund-type adjuvant (Difco). Initial injections were usually 0.1 ml, and the volume was doubled at each successive injection. When test bleedings demonstrated satisfactory antibody levels, the rabbits were exsanguinated and the serum was separated after holding the clots at 6 C overnight. The serum was heated for 30 min at 56 C and passed through a column of G-25 Sephadex (previously equilibrated with 0.025 M sodium-potassium phosphate buffer) before freezing at -15°C .

Enzyme neutralization tests. The protocol used for enzyme-antibody reactions varied somewhat, depending on the potency of the antiserum and the anticipated unitage of the enzyme preparation being tested. Ideally, enzyme and antiserum were mixed in a total volume of 2 ml. The mixture remained at room temperature for 30 min and was then left at 6 C overnight. Test samples were centrifuged for 30 min at $20,000 \times g$, and the supernatant fluid was carefully removed with Pasteur pipettes. Since enzyme-antibody complexes were enzymatically active, both the supernatant fluid and the resuspended pellet could be assayed. Neutralization tests were controlled by mixtures of the particular enzyme and either normal rabbit serum or buffer at pH 6.8.

Complement-fixation tests. Protocols used in complement-fixation (CF) tests were similar to those used for bacterial CF tests. Antisheep hemolysin (Difco) was titrated in the presence of a 1:30 dilution of guinea pig complement (Difco) and a 2% suspension of washed sheep erythrocytes. After 1 hr of incubation at 37 C, the hemolysin unit was determined from the highest dilution that produced complete hemolysis of the erythrocytes. In subsequent CF tests, 2 units of hemolysin per test were used. Complement was titrated in the presence of 2 units of hemolysin and a 2% sheep erythrocyte suspension. After 1 hr at 37 C, the least amount of complement which produced complete hemolysis of the erythrocytes was considered to be one exact unit. A full unit was 0.05 ml more than an exact unit. Two full units of complement were used in subsequent CF tests.

Complete CF tests were run by first mixing the

antigen (enzyme) with an excess of antisera. Two full units of complement were then added, and the tests were incubated for at least 12 hr at 6 C. The tests were then left at room temperature for 15 min before addition of the indicator system consisting of 2 units of hemolysin (in 0.5 ml) and 0.5 ml of a 2% sheep erythrocyte suspension. Final volumes were 3 ml per test, and 0.85% saline, with 0.01% magnesium sulfate incorporated, was used for necessary dilutions of any ingredient. The tests were incubated at 37 C for 1 hr and then centrifuged at $500 \times g$ for 15 min to remove remaining intact blood cells. The supernatant fluids were diluted 1:10 with distilled water; and the optical densities (OD) of the released hemoglobin were read at 414 m μ in a Beckman spectrophotometer. To correlate hemoglobin directly with complement fixation, results were recorded as differences between the OD of the individual tests and the OD of an antiserum control. Usual controls for anticomplementary activity and nonspecific hemolysis were included with each set of CF tests.

Density gradients. Linear sucrose gradients were made in Lusteroid tubes with an apparatus similar in design to one described by Britten and Roberts (3). By use of this device, 2.3 ml of cold 20% sucrose (Certified Reagent, Fisher Scientific Co., Pittsburgh, Pa.) solution was mixed with the same volume of a 5% solution. Sucrose solutions were calculated on a weight per volume basis, and the solvents were a variety of different buffers. Gradients were allowed to stabilize at 6 C for at least 4 hr before use. Preparations to be studied were carefully layered in minimal volumes on the gradients and were centrifuged at 2 C in a Spinco model L machine (Beckman Instruments, Inc., Fullerton, Calif.) at 37,000 rev/min for various lengths of time. The SW-39 swinging bucket rotor was used. After centrifugation, the tubes were punctured on the bottom, and the contents were collected in either 10- or 15-drop fractions.

Enzyme assays. β -Glucosidase was assayed by use of the chromogenic substrate, *p*-nitrophenol- β -D-glucoside (PNPG). Details of the assay procedure have been reported (11).

Lysozyme and catalase were used as markers in some of the density-gradient centrifugation studies. Lyophilized egg white lysozyme (grade 1, three times crystallized; Sigma Chemical Co., St. Louis, Mo.) was dissolved in water to a stock concentration of 10 mg/ml for layering on sucrose gradients. Activity in resultant fractions was assayed at 30 C by noting decreases in OD at 600 m μ of a suspension of *Bacillus megaterium*. Reaction mixtures contained 0.05 ml of enzyme, 0.2 ml of the indicator bacterium, and 2.5 ml of a 0.1 M solution of ethylenediaminetetraacetic acid (EDTA) in 0.85% saline (pH 8.0). A unit of lysozyme was considered to be that amount which reduced the OD by 0.001 per minute per 2.75 ml of reaction mixture.

Lyophilized beef liver catalase (Caperase) was obtained from Laboratorio P.E.V.Y.A. (Barcelona, Spain) and reconstituted with water to a stock concentration of 1,000 labeled units per ml. Usually, 200 units were used for each sucrose gradient, and activity was detected in collected fractions at 30 C by

decreases in the absorbancy at 240 $m\mu$ of a solution of hydrogen peroxide (5). Reaction mixtures contained 0.05 ml of enzyme, 0.2 ml of a 0.375% water solution of hydrogen peroxide (Welton Laboratories Inc., Newark, N.J.), and 2.5 ml of 0.05 M phosphate buffer at pH 7.5. A unit of catalase activity was defined as that amount necessary to cause a change of 0.001 in absorbancy per minute per 2.75 ml of reaction mixture.

Molecular weight determinations. Approximate molecular weights were determined by a method described by Martin and Ames (17). The method employs the equation, $s_1/s_2 = MW_1^{2/3}/MW_2$, which assumes that most enzymes are globular and that their migration through sucrose gradients (of the type used in our studies) is essentially linear. Under these conditions, the relationship between sedimentation constants (s) and molecular weights (MW) expressed in the equation is approximately correct. The distances traveled from the gradient meniscus by the unknown enzyme activity and the marker enzyme can be substituted for the sedimentation constants. For purposes of calculation, the lysozyme marker was assumed to have a molecular weight of 17,200, and the catalase a molecular weight of 250,000.

RESULTS

Although we originally thought that enzyme preparations from the hybrid Y-42 yeast contained two distinct molecular species of β -glucosidase (8), we were never successful in attempts to separate these activities—despite the fact that a mixture of Y-18 and Y-19 enzymes could be resolved with DEAE cellulose. The top portion of Fig. 1 shows that the enzymes were eluted at different molarities of sodium chloride. Identity of the enzymes was established by neutralization tests with specific Y-19 antiserum. When Y-42 enzyme was chromatographed under precisely identical conditions, the results shown in the bottom portion of Fig. 1 were obtained. Enzyme activity was eluted as a single broad peak with some amount of trailing. It is obvious that the total Y-42 activity overlaps the Y-18 and Y-19 activities, and that the peak activity fractions lie between those of the enzymes from the parental yeast strains. The small amount of activity in the later fractions that could not be neutralized will be discussed later.

When the β -glucosidases were subjected to electrophoresis through strips of cellulose polyacetate (Sephraphore III), it was again noted that the Y-42 activity tended to overlap or span the activities of the Y-18 and Y-19 enzymes. Figure 2 presents, schematically, typical migration patterns for the enzymes run simultaneously in a common chamber. The Y-18 enzyme was located in a narrow band 4.1 to 4.4 cm from the point of application, whereas the Y-19 enzyme migrated 3.2 to 3.4 cm from the origin. A mixture of the

FIG. 1. DEAE cellulose chromatography of yeast β -glucosidases. Columns were equilibrated with 0.05 M phosphate buffer (pH) 6.5. The enzymes were eluted with a sodium chloride gradient from 0.15 to 0.4 M. Fractions of 5.5 ml were collected and assayed for enzyme activity by the discontinuous method. Neutralization tests were performed with Y-19 antiserum (19 AS) only.

FIG. 2. Schematic representation of electrophoretic patterns of β -glucosidases. Samples were applied to 1 by 6.75-inch strips of cellulose polyacetate and allowed to migrate for 70 min at 200 v (Heathkit Power Supply) in TSM buffer (pH 7.2). The patterns were developed by spraying the strips with PNPG in 0.05 M phosphate buffer (pH 7.0), followed a few minutes later by spraying with 1 M sodium carbonate. The dashed lines represent the areas of sample application.

Y-18 and Y-19 enzymes resolved into two narrow bands 3.9 to 4.2 and 3.2 to 3.5 cm from the point of application. The Y-42 enzyme preparation migrated as a single broad zone and was located 3.0 to 4.1 cm from the origin.

When potent antisera against both the Y-18 and Y-19 enzymes were available, it was possible to study the Y-42 system more definitively. Specificity of the enzyme-antiserum reactions is shown in Fig. 3 by a common neutralization slope for the homologous systems and the complete lack of neutralization in heterologous systems. It was enlightening to find that *either* antiserum could neutralize the major portion of Y-42 enzyme activity. The extent of neutralization varied somewhat, depending on the particular batch of antiserum used, but was 80 to 90% of the Y-42 activity. Importantly, it was also found that the enzyme activity remaining after treatment with either the Y-18 or Y-19 antiserum was essentially completely neutralized by the opposite antiserum. It should be added, parenthetically, that we refer to enzyme-antibody interactions as enzyme neutralization. Immunologically, the interaction is more accurately a precipitin type. The antigen (enzyme) is soluble, a visible precipitate forms upon the addition of homologous antiserum, and there appears to be zoning in antigen or antibody excess.

FIG. 3. Neutralization of β -glucosidases with antisera. Reaction mixtures usually contained 300 units of enzyme (E) plus the indicated quantities of antiserum (AS). After the mixtures were processed as described in Materials and Methods, residual enzyme activity was assayed by the discontinuous method. Controls were enzyme alone in TSM buffer (pH 6.8), and were processed exactly like the test mixtures.

To substantiate the proposed hybrid nature of Y-42 preparations, many attempts were made to dissociate the enzymes and identify the products. If such treatment served to resolve the hybrid enzyme molecules into their constituent "parental" subunits, it was reasoned that specific antiserum might precipitate one of the parental activities and demonstrate a concomitant increase in the other parental activity. By heating Y-42 enzyme at temperatures below those which caused a rapid decline in enzymatic activity (50 to 52 C), we demonstrated a modest increase of 14% in activity that could not be neutralized with Y-18 antiserum. Figure 4 shows the results of a more elaborate experiment. The enzyme was dissociated with urea, and neutralization tests were performed with both Y-18 and Y-19 antisera. The significant aspect of Fig. 4 is that both parental activities increased during the time-course of the experiment.

Attempts to study the dissociation products of Y-42 enzyme emphasized the need for a technique of identification which was not based on enzymatic hydrolysis of PNPg. Dissociation of enzyme molecules infers that their structural integrity is destroyed and, correspondingly, their

FIG. 4. Dissociation of Y-42 enzyme with urea. Enzyme was exposed to 4 M urea at 30 C. Periodically, samples were withdrawn and diluted highly in 0.05 M phosphate buffer (pH 6.8). The resultant pools were used for enzyme assays and neutralization tests with both Y-18 and Y-19 antisera. Percentages of activity that could not be neutralized were calculated on the basis of total enzyme activity in the original dilution pools. Controls consisted of enzyme which was not exposed to urea, but was subjected to neutralization tests. By dividing percentages of activity obtained from the urea-treated samples which could not be neutralized by those obtained from the controls, results were normalized and could be compared directly.

ability to hydrolyze substrate. We found that β -glucosidase-antibody complexes would bind guinea pig complement and that a standard CF test was useful for monitoring dissociation studies. The fixation of complement by enzyme-antibody complexes displayed the same specificity patterns as did neutralization tests. In comparative studies, it was also found that the CF reaction was more stable than enzymatic hydrolysis; i.e., the CF test could monitor dissociation of the enzymes beyond the point at which hydrolytic activity was lost.

Table 1 shows typical results obtained by using the CF test to identify dissociation products of Y-42 enzyme. In this experiment, the enzyme was quickly heated to 56 C and small portions were subsequently removed for addition to cold Y-18 antiserum. For control purposes, equal portions were also withdrawn and added to cold TSM buffer. After overnight refrigeration, the samples were tested for CF activity with Y-19 antiserum. Considering the highest dilution of antigen capable of complete fixation of complement (no hemolysis) as its titer, the results indicate a twofold increase in specific Y-19 activity. Urea was also used as the dissociating agent in other studies of this type, and the same results were obtained.

The constituent parental subunits of Y-42 enzyme were apparently too similar to be resolved by centrifugation of dissociated preparations in sucrose gradients. Studying the dissociation products of Y-18 and Y-19 enzymes, however, gave us additional insight into the molecular structure of the yeast β -glucosidases. Figure 5 shows the type of results which suggested that the native enzymes were composed of three or four structural subunits. The intact enzyme peak and the subunit peak were calculated to have molecular

FIG. 5. Density gradient centrifugation of urea-dissociated Y-18 enzyme. The enzyme was exposed to 4 M urea for 12 min at 30 C, after which it was diluted in 0.05 M phosphate buffer (pH 6.8). A small sample (0.35 ml) was layered on a sucrose gradient made in 0.05 M phosphate buffer. A second identical gradient was layered with a mixture of catalase and lysozyme. The gradients were centrifuged for 16 hr at 37,000 rev/min, after which 15-drop fractions were collected. Fractions from the first gradient were assayed for β -glucosidase and tested for CF activity with Y-18 antiserum. Fractions from the second gradient were assayed for the marker enzymes as described in Materials and Methods.

weights of 325,000 and 110,000, respectively. The very heavy CF particles found in the early fractions may have been aggregates (21, 27). When the time of exposure to urea was lengthened to 40 min and 0.5 M urea was incorporated with the sucrose gradients, different results were obtained. As many as four peaks of CF activity were observed, the lightest corresponding to molecular weights ranging from 18,000 to 23,000. These light-weight dissociation products were eventually demonstrated with both enzymes from the parent yeast strains and with the hybrid yeast enzyme as well, when gradient fractions of the latter were tested with either parental antiserum.

Figure 6 shows the multiple bands of CF activity which were resolved by gradient centrifugation of Y-42 enzyme after prolonged exposure to urea. Although activity was of low order, the results indicated that several different sizes of particles were produced. The reason for the native enzyme peak not coinciding more closely with one of the CF peaks is not known.

The need for a low concentration of urea in sucrose gradients to demonstrate the small CF particles suggested that the dissociated subunits might tend to reassociate under certain conditions. This possibility was investigated by use of urea-dissociated Y-42 enzyme. The level of regenerated activity was low, but the results suggested that several factors might influence the

TABLE 1. Dissociation of Y-42 enzyme with heat

Sam- pling times	CF titrations of parental Y-19 activity						
	Controls			Tests			
	1:32	1:64	1:128	1:32	1:64	1:128	1:256
min							
0 ^a	— ^b	+	+	—	—	—	+++
5	—	+-	++	—	—	+	++
10	—	+	++	—	—	+-	++
15	—	+	++	—	—	+	+++
20	—	++	++	—	—	++	+++
30	—	++	+++	—	+-	++	+++

^a Samples withdrawn as quickly as possible after enzyme preparation reached 56 C.

^b Degrees of hemolysis: no hemolysis indicates complete fixation of complement and 4+ hemolysis indicates no fixation of complement.

extent of reassociation of the enzyme subunits. A pH of 6.5 seemed more favorable for reassociation than pH 7.5. A low molarity phosphate buffer (0.01 M) was superior, but reassociation in 0.1 M citrate was better than in 0.01 M citrate. Table 2 shows that, in either buffer system, the amount of

residual urea affects the level of regenerated enzyme activity. The presence of 2-mercaptoethanol seemed to enhance reassociation.

DISCUSSION

Allen et al. (1) proposed that true hybrid enzymes are forms produced as a result of interaction between alleles or nonalleles. Descriptions of such systems are not uncommon and have been reported for bacteria, fungi, higher plants, birds, and mammals (2, 4, 10, 15, 16, 25, 28). A system studied by Markert (16) is particularly apropos to a discussion of our own results. Five different molecular forms of tetrameric lactate dehydrogenase (LDH) are present in beef tissue, and the constituent subunits exist in two genetically determined electrophoretic varieties. The different forms of the enzyme are thought to represent all possible combinations of the subunits. The LDH-1 and LDH-5 forms are apparently constructed solely of one or the other type of subunit and can be separated by DEAE chromatography. Cahn et al. (4) studied the same enzyme in chickens. During embryonic development, there is a gradual shift through several intermediate hybrid enzyme types from a pure LDH form found in heart muscle to a pure form found in breast muscle. Antiserum against either pure enzyme reacts with the hybrid enzymes in precipitation or CF tests.

Our results, in general, are best explained by assuming that the β -glucosidase activity in Y-42 preparations is due primarily to a population of different hybrid enzyme molecules. Also, there is reason to believe that the hybrids consist of various combinations of two distinct polypeptides which are characteristic of those produced by the parental Y-18 and Y-19 yeast strains. If the hybrids were formed by random association of the two subunit types, a binomial distribution would dictate the presence of a few pure parental enzyme molecules.

The hybrid nature of Y-42 enzyme was indicated by results presented in this and the preceding paper. Characteristic values for Y-42 enzyme were found intermediate to, or spanning, those of the parental enzymes. Molecular weight determinations for the smallest particles resolvable from the enzymes by urea treatment would suggest that the native enzyme molecules could consist of 12 or more subunits. If such were the case, separation of the hybrid molecules in Y-42 preparations would be difficult, and one might expect a diffuse activity similar to that shown in Fig. 2. Although the results were too fragmentary to include in this report, we were able to resolve partially purified Y-42 enzyme into at least 10 distinct bands with polyacrylamide gel (19). A

FIG. 6. Density gradient centrifugation of urea-dissociated Y-42 enzyme. The enzyme was exposed to 4 M urea for 40 min at 30 C, after which it was diluted in 0.05 M phosphate buffer (pH 6.8). A small sample was then centrifuged for 12 hr at 37,000 rev/min on a sucrose gradient. The gradient was made in TSM buffer (pH 6.8), and contained 0.5 M urea. The 15-drop fractions were subsequently assayed for β -glucosidase and for CF activity, using only Y-18 antiserum.

TABLE 2. Effects of residual urea and mercaptoethanol (ME) on the regeneration of enzyme activity after urea-dissociation of Y-42 enzyme^a

Buffer system	Residual urea	ME	Regenerated enzyme (% of control) ^b
0.01 M phosphate, pH 6.5	M	M	
	0.5	—	12
	0.25	—	16
0.1 M citrate-phosphate, pH 6.4 ^c	0.5	—	12
	0.25	—	14
	0.125	—	17
0.01 M phosphate, pH 6.5	0.125	0.01	21
	0.125	0.1	25

^a Enzyme was exposed to 4 M urea for 10 min before dilution to the indicated residual urea concentrations.

^b Separate controls were run with each test and manipulated in the same manner, except for the initial exposure to urea and the addition of ME. Samples were assayed by the discontinuous method after 20 hr of storage at 6 C.

^c Molarity based on citrate component.

number of these bands demonstrated enzyme activity.

The expectation that Y-42 preparations should contain low levels of pure parental enzyme activity was borne out by the results shown in Fig. 1. The small amount of enzyme activity which was not neutralized with Y-19 antiserum undoubtedly represented pure Y-18 enzyme. If the DEAE fractions had been tested with Y-18 antiserum, a similar area of Y-19 activity that could not be neutralized would probably have been detected on the left-hand side of the broad elution peak. These results were essentially the same as those obtained by continuous-flow electrophoresis (8). The strongest evidence for the heterogeneous population of enzyme molecules from Y-42 was serological data. Activity that could not be neutralized with either parental antiserum most certainly represented enzyme molecules containing a preponderance of the opposite parental subunit.

Results obtained from studying the dissociation products of Y-42 enzyme (Fig. 4 and Table 1) suggest the presence of two different subunit types in Y-42 preparations. It could be argued that urea or heat treatment simply served to alter the configuration of the enzyme molecules (without actual dissociation to parental subunits) and that this, in turn, was demonstrable by increases in activity that could not be neutralized with either parental antiserum. However, activity that could not be neutralized was detectable in the supernatant fluids of these tests, and calculations were based on total enzyme activity in controls which were also exposed to the dissociating agent, but not mixed with antiserum. If simple distortion of the Y-42 molecules occurred, activity that could not be neutralized would not have increased relative to the controls, but would have remained at the same level as the controls. Moreover, we know from sucrose gradient studies that Y-42 enzyme is broken into smaller pieces by the conditions used for dissociation.

Reversible dissociation of enzymes has been abundantly demonstrated (7, 9, 16, 18, 20, 23, 24). Although we were not able to demonstrate the regeneration of large amounts of activity after dissociation of yeast β -glucosidase, our results suggested that pH, ionic strength of the buffer system, and residual dissociating agent were conditions that influence the extent of subunit association. Markert (16) felt that urea dissociation of lactate dehydrogenase was too severe to permit subsequent reassociation of monomers. Perrin and Monod (20) compared the extent of reassociation of chemically dissociated β -galactosidase, and found that more enzyme activity was recovered when the dissociating agent was slowly

removed by dialysis than when the concentration of the agent was reduced suddenly by high dilution with buffer. These investigators theorized that rapid removal of the dissociating substance did not allow sufficient time for the proper bonds to be formed in the proper order.

It was mentioned earlier that multiple bands of CF activity were obtained by sucrose-gradient centrifugation of enzyme preparations after sufficient exposure to urea. It seems reasonable to assume that the light particles (molecular weight of 18,000 to 23,000) were demonstrable because reaggregation was inhibited to some degree during centrifugation by the presence of 0.5 M urea in the gradients. Heavier particles may have represented either residual dissociation products or aggregates of the light particles. Protocols used for CF tests subjected the gradient fractions to at least a 1:20 dilution, which would affect a considerable reduction in the concentration of residual urea. Occasionally, we were able to demonstrate small amounts of β -glucosidase activity in CF fractions which had been so diluted and incubated at 6°C for several hours in the presence of PNP-G. Although the ability to fix complement was demonstrated to be a more stable feature of the yeast β -glucosidases than their hydrolytic capacity, it seemed improbable that serological specificity could remain intact after dissociation of the molecules to their smallest subunit particles. In connection with studies on the dissociation of Y-42 enzyme by extreme pH (8.5), a small complement-fixing particle (molecular weight, 17,000) was obtained by filtration through G-100 Sephadex and was subsequently run on a sucrose gradient. The activity was found in precisely the same fraction that contained native enzyme in a parallel gradient.

It is known that different types of control mechanisms are operative in yeast for the synthesis of enzymes. Herman and Halvorson (14) identified a single gene locus which was responsible for the structure of β -glucosidase in *S. lactis*, and they maintained that there were two regulatory genes involved in the control of synthesis of this enzyme. Winge and Roberts (29) first reported the situation wherein polymeric genes were involved in the synthesis of α -glucosidase in various *Saccharomyces* yeasts. Rudert and Halvorson (22) studied the α -glucosidase system, and demonstrated that the level of enzyme production in hybrids was proportional to gene dosage. Since different levels of enzyme were produced by the polymeric genes, they felt that each was under the control of a closely linked operator gene and that the individual operator genes demonstrated different affinities for a single repressor system.

S. fragilis and *S. dobzhanskii* are homothallic yeasts and consequently are not amenable to genetic analysis. We have seen that the β -glucosidases produced by these yeasts differ significantly with regard to several characteristics which involve the superficial structure of the molecules, e.g., serological specificity, affinity constants, and electrophoretic mobility. Since it is generally believed that the amino acid sequences in polypeptide chains of a protein dictate its secondary and tertiary structures, it would seem safe to assume that the enzymes from the parental yeast strains are products of nonallelic genes. Halvorson et al. (13) stated that, since the regulatory behaviors (induction and repression) of the Y-18 and Y-19 yeasts were indistinguishable, the two structural genes were probably under similar regulatory control. Gorman et al. (12) studied the synthesis of β -glucosidase in synchronized cultures of Y-42. That synthesis of two different types of polypeptide chains is ordered in this system was supported by the observation that two distinct periods of enzyme synthesis occurred during each cell generation time. These investigators felt that their results indicated that two nonallelic structural genes for β -glucosidase were present and that transcription of each was nonrandom. A disconcerting aspect of the report by Gorman et al. (12) was the inference that the two bursts of synthesis in Y-42 cells represented the synthesis of Y-18 and Y-19 enzyme. We must assume from our studies, however, that such periods of intermittent synthesis correspond to the appearance of a population of enzyme molecules—the majority of which are hybrids composed of varying proportions of two different types of polypeptide.

The rates of synthesis of Y-18 and Y-19 enzyme are quite different (8). Grossly, the total differential rate of synthesis of β -glucosidase activity by Y-42 appears to be equal to the sum of the differential rates of enzyme synthesis by the parental strains. If we assume that a given population of Y-42 enzyme molecules contains equal numbers of the two parental subunits or monomers, it may be necessary to invoke a unique regulatory mechanism. Colombo and Baglioni (6) recently proposed a mechanism for synthesis of hemoglobin. They suggested that the rate of formation or availability of the β -chains regulated the release of α -chains from polysomes. Similar but more conclusive results by Shapiro et al. (26) have shown that the synthesis of light and heavy chains of γ -globulin occurs on separate, distinguishable polysomes. Assembly appears to occur by attachment of the free, released light chains onto the nascent heavy chains.

Collecting our comments, we would say that

the structural genes for Y-18 and Y-19 β -glucosidase are present in Y-42 cells. Either closely interacting or independent regulatory mechanisms result in unequal synthesis of the parental-type polypeptides. On or near the polysomes, available parental subunits are equalized by some unknown process, and random polymerization occurs in the cell cytoplasm to produce a binomial distribution of hybrid enzyme molecules.

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