*ANTIBODY-MEDIATED ACTIVATION OF A DEFECTIVE β-D-GALACTOSIDASE EXTRACTED FROM AN ESCHERICHIA COLI MUTANT**

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The interaction of β-D-galactosidase with its specific antibody does not lead to any significant change in enzymatic activity regardless of whether or not immune precipitation occurs.[1] Cowie *et al.*[2] found that ribosome-bound β-D-galactosidase differed from soluble enzyme in that an increase in enzymatic activity occurred in the presence of anti-β-D-galactosidase serum. This was ascribed to the formation of new centers of β-D-galactosidase activity on the ribosomes.[3] We were interested in these observations for their potential use in a system to detect antibodies because the appearance of enzymatic activity would be a signal with inherent amplification capabilities.

The antibody activation of ribosomal enzyme in the wild-type *Escherichia coli* was not readily amenable to our purposes because the average gain in activity was only threefold. We conducted a search for larger activations by screening a series of point mutants known to be defective in the *z* gene. Out of 47 tested, 2 were found.

A biochemical characterization of an enzyme precursor found in the extract of one of these two mutants is presented below. This precursor is referred to as AMEF (antibody-mediated enzyme-forming substance).

Materials and Methods.—(a) *Purification of β-D-galactosidase (β-D-galactoside galactohydrolase E.C. 3.2.1.23)*: Cells of K12 3300, grown in a 500-liter batch, were used to prepare purified β-D-galactosidase according to Craven *et al.*[4] The specific activity of our enzyme was comparable to that of a sample (a gift of Dr. Steers) estimated to be homogenous by several criteria.[4] The enzyme was stored in a refrigerator under a 40% saturated solution of ammonium sulfate in buffer B. Buffer B, which was used throughout this work, contained 0.01 *M* tris(hydroxymethyl)aminomethane acetate, 0.01 *M* $MgCl_2$, 0.1 *M* NaCl, and 0.05 *M* 2-mercaptoethanol. The complete buffer was prepared daily by adding mercaptoethanol to the salts solution; its pH was 7.05 (23°).

(b) *Anti-β-D-galactosidase serum:* Each of eight rabbits was injected subcutaneously in the scapular region with 25 mg of purified enzyme emulsified in 2 ml of Difco incomplete Freund's adjuvant. A similar injection of 15 mg of antigen was given after 85 days. Twenty-two days later, the animals were bled by heart puncture, and their sera were assayed for precipitins, pooled, and quick-frozen in 1-ml aliquots. One ml of diluted antiserum titrated against 103 units of purified β-D-galactosidase, corresponding to 0.18 μg of enzyme, gave a steep precipitation curve reaching an equivalence zone at an antiserum dilution of 1:2000.

(c) *Screening for antibody-activatable precursors in mutants:* The majority of the strains were point mutants that had been previously identified as being defective in the *z* gene.[5] Cells were grown overnight at 37° to stationary phase in 30 ml of Davis minimal medium,[6] containing 0.4% sodium lactate and 5×10^{-4} *M* isopropyl-1-thio-β-D-galactopyranoside (IPTG). The bacteria were centrifuged down at 12,000 × *g* for 10 min, resuspended in 3 ml of buffer B, and disrupted in a French pressure cell. To assay the resulting extract, samples of 0.5 ml were mixed with 0.1 ml of either normal rabbit serum, anti-β-galactosidase serum, or saline. After 30-min incubation at 37°, 0.1 ml from each mixture

was assayed for β-D-galactosidase activity. No significant activation by antibody was observed when either intact cells or toluene-treated cultures were used instead of extracts.

(d) *Preparation and purification of extracts containing AMEF:* Cells of W6101 (F^- *Lac201*)[5] were grown under the same conditions indicated above for screening, except that the volume of the culture was increased to 15 liters. The cells were washed twice with buffer B, resuspended in 50 ml of the same buffer, and lysed in a French pressure cell. The extract was centrifuged once at 15,000 × *g* for 15 min and then at 70,000 × *g* for 85 min. The resulting supernatant was used as the source of AMEF in several experiments. Further purification involved precipitation with ammonium sulfate (pH 7.2) at 20% saturation, elimination of the precipitate, precipitation of the supernatant at 33% saturation, dissolution of the precipitate in 7 ml of buffer B, and immediate separation by chromatography with buffer B through a Sephadex G10 column (2.7 × 32 cm) with an input of 3.5 ml. Fractions containing AMEF, which were found very near the front, were pooled, precipitated with ammonium sulfate at 33% saturation, redissolved, and either dialyzed or passed through a column of Sephadex G10. This procedure yielded preparations with 30–40 times higher specific activity than that of the crude original extract.

(e) *AMEF assay:* Mixtures of 0.1 ml of the test preparation plus 0.1 ml of a given dilution of antiserum (all dilutions were made with buffer B) were incubated at 37°, and samples withdrawn at intervals for β-D-galactosidase assay. Controls contained normal rabbit serum instead of antiserum. A 1:10 dilution of antiserum and 30 min incubation at 37° were used for routine testing.

For β-D-galactosidase assays, the test solution (in a volume of 0.04 to 0.1 ml) was added to a tube equilibrated at 37° containing a solution of o-nitrophenyl-β-D-galactopyranoside (ONPG) in buffer B to make a total volume of 2 ml and a final substrate concentration of 3×10^{-3} *M*. The tubes were kept at 37° for less than 10 min until a visible yellow color developed; then 3 ml of 0.2 *M* Na_2CO_3 was added to stop the reaction and the optical density at 420 mμ was measured. A molar extinction coefficient (1-cm light pathway) of 4700 was used to convert readings to o-nitrophenol concentrations. One β-D-galactosidase unit is defined as the amount of enzyme liberating 10^{-9} moles of o-nitrophenol in 1 min. Immunodiffusion experiments were done according to the Ouchterlony procedure.[8]

(f) *Assays at the single molecule level:* The molecular enzymatic activity of AMEF was compared with that of normal β-D-galactosidase by means of the fluorogenic assay.[12, 13] The purified extract containing AMEF was mixed with limiting amounts of antiserum in order to obtain activation in antigen excess and thus avoid immune precipitation. The enzymatic activity of this mixture measured by the routine ONPG assay was adjusted by dilution to match that of a normal β-D-galactosidase solution. Each solution was then mixed with fluorescein-di-(β-D-galactopyranoside) and dispersed into microdroplets so as to obtain an all-or-none distribution of enzyme with an average of 0.67 molecule per microdroplet of 20.34-μ/diameter. After 16 hr of incubation at 35°, the fluorogenic activity of at least 60 individual microdroplets per slide was measured.

(g) *Chemicals:* 2-Mercaptoethanol was a product of Eastman Kodak. The inducers, substrates, and yeast lactase were obtained from Mann Laboratories, Inc. Bacterial alkaline phosphatase, beef catalase, deoxyribonuclease I, and trypsin were purchased from Worthington, Inc., and pancreatic ribonuclease-A from Sigma, Inc.

Results.—Extracts from induced cultures of 47 point mutants defective in the *z* gene[5] were screened for enhancement of their β-D-galactosidase by anti-β-D-galactosidase serum. Thirty-seven of the mutants exhibited no significant enhancement (less than twofold). Six had enhancement factors between 2 and 4, two had factors of 9 and 23, respectively, and two had factors over 100. The last two did not appear to differ in either genetical or biochemical tests. Of these two, W6101 (F^- *Lac201*) was chosen for the present studies.

TABLE 1. *Specificity of the antibody-mediated enzyme formation.*

Type of preparation	Type of serum	β-D-galactosidase (units/ml)
AMEF	Anti-*coli* β-D-galactosidase	6750
AMEF	Normal	34
AMEF	None	29
Buffer B	Anti-*coli* β-D-galactosidase	0
AMEF	Anti-yeast β-D-galactosidase	36
AMEF (preheated 8 min at 60°)	Anti-*coli* β-D-galactosidase	7
AMEF	Purified γ-globulin	4680
AMEF	Absorbed antiserum	113
Buffer B	Absorbed antiserum	20
β-D-galactosidase	Normal	385
β-D-galactosidase	Anti-*coli* β-D-galactosidase	292

A crude preparation of AMEF was assayed with different sera as described under *Materials and Methods*. All sera were diluted 1:10. In addition, the anti-yeast β-D-galactosidase serum was assayed at 1:2 dilution, giving 31 units/ml. The γ-globulin was purified from antiserum by starch electrophoresis. The absorbed antiserum was the supernatant remaining after 1 ml of antiserum was precipitated at equivalence point with purified β-D-galactosidase.

In contrast to earlier findings in wild-type *E. coli*,[2, 3] the enhancement caused by antiserum in W6101 was demonstrable both in the ribosomal and in the soluble part of the cell extract. We elected to use the soluble part of the extract for the biochemical characterization of the molecular species (AMEF) activated by the antiserum.

Table 1 shows that after incubation with anti-β-D-galactosidase serum, the enzymatic activity of a crude preparation of AMEF in contrast to that of normal enzyme, was 170 times higher than that of controls and that the increase was immunologically specific. Preparations of γ-globulin purified by either electrophoresis or ammonium sulfate precipitation of antiserum were as active as whole antiserum. Antiserum absorbed with purified β-D-galactosidase showed reduced reaction with AMEF. The complete cross reaction of AMEF and wild-type β-D-galactosidase was also demonstrated by bidimensional immunodiffusion preparations using the Ouchterlony method. Predigestion of AMEF preparations with trypsin (0.5 mg/ml, 30 min at 37°) resulted in a 90 per cent loss of the capacity to yield enzymatic activity after treatment with antiserum (the trypsin concentration was diluted 1:400 prior to the addition of antiserum so as not to affect the latter). In contrast, treatment of AMEF with DNAse (10 μg/ml) or pancreatic RNAse (10 μg/ml) caused no detectable loss of activity.

Requirements for the activation of AMEF: The hydrolysis of ONPG in the presence of antibody-activated AMEF proceeded at a constant rate for the first 15 minutes. Slightly higher rates were observed after this time. Accordingly, routine assays were not extended beyond 10 minutes. Under these conditions, the rate of ONPG hydrolysis was found to be proportional to the amount of activated AMEF present in the assay.

Induction of AMEF: The biosynthesis of AMEF was induced specifically by the same compounds known to induce β-D-galactosidase biosynthesis (Table 2). In the presence of 10^{-3} *M* IPTG, the cells synthesized AMEF at a differential rate of 40–100 enzyme units per 0.1 unit of increase in optical density (650 mμ) of the culture. The rate remained constant after an initial lag, which was found to be variable from experiment to experiment.

TABLE 2. *The specificity of induction of AMEF biosynthesis.*

Inducer	β-D-Galactosidase Activity: Antiserum added	β-D-Galactosidase Activity: No antiserum added
None	0.7	<0.05
Isopropyl-1-thio-β-D-galactopyranoside (IPTG)	690	3.6
Methyl-1-thio-β-D-galactopyranoside (TMG)	380	1.9
Lactose	2	<0.05
β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG)	5	<0.05
Phenyl-β-D-galactopyranoside	0.6	<0.05
D-fucose	10	<0.05

Cells of W6101 were grown for 3.5 hr in the presence of 10^{-3} *M* inducer. The cells were harvested, washed, extracted, and their β-D-galactosidase activity was determined either directly or after incubation with antiserum, as described in the text. The activity is expressed in enzyme units per optical density unit (280 mμ) of the cell-free extract.

The amount of AMEF per cell synthesized by W6101, as measured by its enzymatic activity after exposure to antiserum, was about 10 per cent of the β-D-galactosidase activity present in the wild-type strain of K-12 (W3110).

Purification and estimation of molecular weight of AMEF: A 30-fold purification of AMEF was achieved (Table 3) by a series of steps resembling those used by Craven *et al.* for β-D-galactosidase.[4] The purified fractions were stable at 4° for at least three weeks if kept undiluted. The most successful purification steps were those using ammonium sulfate precipitation followed by gel filtration through Sephadex G10 or G200. Reversible losses were frequently observed during gel filtration. The fractions obtained from the Sephadex columns were assayed, pooled, and precipitated with 33 per cent ammonium sulfate as quickly as possible in order to avoid inactivation.

A sedimentation pattern of a purified AMEF preparation in a sucrose gradient is shown in Figure 1. Normal β-D-galactosidase and bacterial alkaline phosphatase were used as reference standards. Using a reference value of $s^{0.725}_{20,w}$ equal to 16.0 for β-D-galactosidase,[4, 9] the average $s^{0.725}_{20,w}$ of AMEF was 15.1 and that of alkaline phosphatase 6.0. A sedimentation coefficient of 6.3 has been reported for the latter measured under different conditions.[10] The "native" enzymatic activity present in the extracts of W6101 sedimented at the same rate as AMEF. If it is assumed that the AMEF molecule has the same shape as that of normal β-D-galactosidase and that the latter has a molecular weight of 540,000,[4] the

TABLE 3. *Purification of AMEF.*

Step	Specific activity (enzyme units per OD_{280} unit)	Per cent activity recovered in step
Soluble extract	1,780	
$(NH_4)_2SO_4$ ppt (20–33% cut)	22,200	78
Sephadex G10	18,550*	42*
33% $(NH_4)_2SO_4$ ppt	46,600	67
Sephadex G200	75,100	46

See section on *Materials and Methods* for details. Fifty-five ml of soluble extract with an AMEF activity of 3.2×10^5 units per ml was used as starting material.

* We often observed a loss of activity after Sephadex G10 fractionation, which was reversed after ammonium sulfate precipitation.

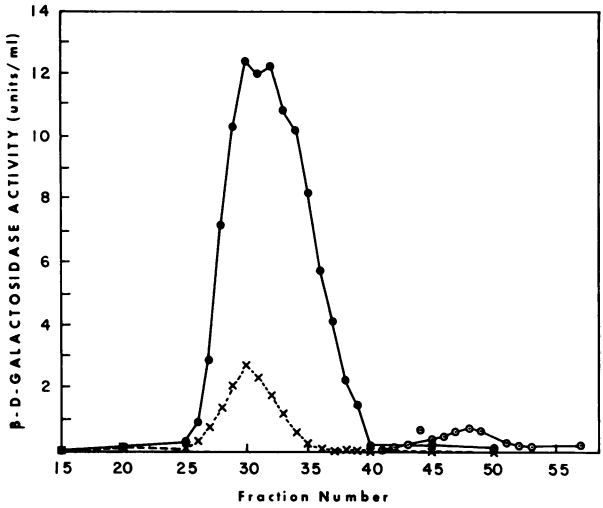

FIG. 1.—Sedimentation pattern of a purified preparation of AMEF in a sucrose gradient. The method of Martin and Ames[7] was used. Tubes containing samples of 0.65 ml layered on top of preformed gradients of 5–20 % sucrose in buffer B were centrifuged for 21 hr at 24,000 rpm in a SW25 rotor of a Spinco model L. The tubes were then punctured and 20-drop fractions were collected. The fractions were assayed for AMEF activation by antiserum (●—●), bacterial alkaline phosphatase (⊙—⊙), and normal β-D-galactosidase (×—×).

molecular weight of AMEF calculated[7] from the average sedimentation data of three experiments was 495,000.

Estimations of the molecular weight of AMEF by the Sephadex filtration method,[11] using blue dextran, normal β-D-galactosidase, beef catalase, and bacterial alkaline phosphatase as reference standards, indicated that it was above 400,000.

Quantitative aspects of the AMEF reaction with antibody: The results of experiments in which the β-D-galactosidase activity formed after a given amount of AMEF was mixed with increasing concentrations of antibody are illustrated in Figure 2. The data shows: (*a*) In the antigen-excess zone, that is, where more than 95 per cent of the resulting enzymatic activity was in soluble form, the activation was proportional to the antibody concentration. This indicated that the formation of enzymatic activity was independent of the immune precipitation of the reactants. (*b*) At both the equivalence- and antibody-excess zones, the activity reached a maximum, and more than 99 per cent of it was found in the precipitate.

Relationship between AMEF and native enzymatic activity: All extracts of W6101 had some β-D-galactosidase activity in the absence of antibody. In contrast to AMEF, this native activity decreased rapidly during the purification procedure for AMEF and even during storage at 4°. To study the kinetics of inactivation, the preparations were incubated at 37° for a few minutes.

In a series of experiments, a preparation of AMEF was incubated at 37°;

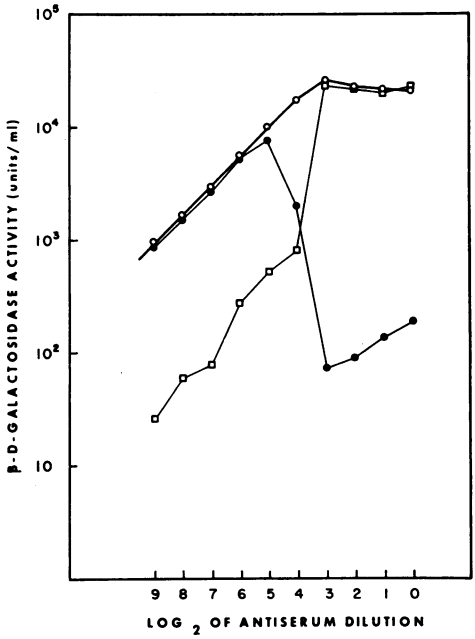

FIG. 2.—Reaction of AMEF and antiserum. Aliquots of purified preparations of AMEF were each mixed with an equal volume of the indicated dilution of antiserum and incubated at 37° for 30 min. After treatment, each AMEF-antibody mixture was chilled, and a portion of it separated by centrifugation into a supernatant and a precipitate. The β-D-galactosidase activity in the total mixture (O—O), in the supernatant (●—●), and in the precipitate (□—□) was measured.

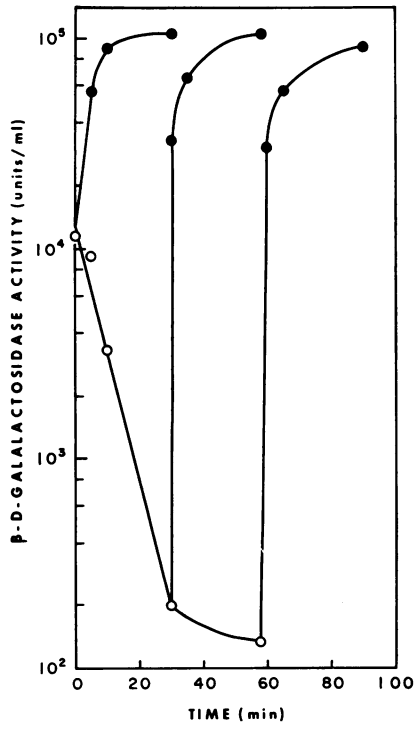

FIG. 3.—Effect of antiserum on extract containing inactivated native β-D-galactosidase. A crude preparation of AMEF mixed with a dilution 1:5 of *normal* rabbit serum was incubated at 37°, and samples were taken at intervals for β-D-galactosidase assay (O—O). Additional samples taken at 0, 30, and 58 min were mixed with equal volumes of a 1:4 dilution of *antiserum*, incubated at 37°, and assayed at intervals in order to measure the increase in enzyme due to AMEF (●—●).

samples were taken at intervals and assayed for native β-D-galactosidase; and their activation in the presence of antibody was followed kinetically. The results of one of these experiments are shown in Figure 3. The native enzymatic activity decreased exponentially in the first 30 minutes with a half life of 6 minutes, whereas the AMEF activity measured after treatment with antiserum remained comparable to that of the zero-time sample.

The exponential decrease in native enzymatic activity at 37° began to level off after 30 minutes, approaching constant values. At these levels, addition of antiserum to the preparation resulted in about a 200-fold increase in enzymatic activity. Some preparations exhibited increases up to 550-fold when the native enzyme was inactivated at lower pH in the presence of 5 per cent bovine albumin.

The results of assays of AMEF-antibody mixtures at the single molecule level

by the fluorogenic method indicated that preparations of AMEF were composed of at least two types of enzymatically active molecules, one more stable during the assay than the other. Neither of these was identical to the molecular species of enzyme present in β-D-galactosidase preparations from normal bacteria.

Discussion.—The aim of this research was to find a system in which a specific antibody reaction could result in a substantial gain of enzymatic activity. We were able to obtain cell-free preparations of *E. coli* that exhibited a 550-fold stimulation of β-D-galactosidase activity in the presence of specific antiserum.

In the systems previously described,[2, 3, 14–16] the enzyme activation in the presence of antibody was comparatively low. Of these systems, the most responsive is that found by Pollock.[15] He reported that the penicillinase activity of a mutant of *Bacillus licheniformis* was enhanced 70-fold by antipenicillinase serum.

From the results given above, it follows that the biosynthesis of AMEF is directed by an altered *z* gene and regulated by the *i* gene of the *Lac* operon. The molecular weight of AMEF is either about 495,000 (92 % of β-D-galactosidase) or is the same as that of β-D-galactosidase (540,000), and conformational changes in the AMEF molecule account for the differences found in sedimentation and gel filtration analyses. Since the normal enzyme consists of four monomers, each with a molecular weight of 135,000, it seems probable that AMEF is also a tetramer. The possibility that AMEF is a trimer is difficult to reconcile with the sedimentation data.

The native enzymatic activity present in AMEF preparations prior to treatment with antiserum could not be separated from AMEF by any of our purification procedures. However, in contrast to AMEF, the native activity was very labile and could be reduced to negligible levels.

With regard to the mechanism underlying the activation by antiserum, one possibility is that it serves to stabilize the native enzymatic activity. However, this hypothesis is ruled out by the results of experiments in which the capacity of a preparation to be activated remained unaffected, whereas the native enzymatic activity was reduced to one thousandth of its original value prior to the addition of antiserum (Fig. 3).

If the β-D-galactosidase molecule is represented according to the model suggested by electron microscopic studies,[17] AMEF can be visualized as a molecule that would undergo conformational changes after contact with antiserum, yielding a quadrangular structure similar to the normal enzyme.

The recombination analysis[5] of W6101 indicated that its altered site is overlapped by the deletion present in W4680 (*Lac39*), an $i^+z^-y^+$ mutant. This deletion has been located in the middle of the *z* cistron and covers about 36 per cent of the gene.[18] These results indicate that an amino acid substitution is responsible for the properties of AMEF, since a chain-terminating mutation near the center of the gene would have given a smaller product[19] than we observed.

The data obtained from the experiments with fluorescein-di-(β-D-galactopyranoside) indicate that the assay with AMEF could be used to measure antibodies at either the cellular or the molecular level. A method capable of measuring approximately 100–200 molecules of antibody has been reported recently.[20]

Its basis is the fluorogenic assay of β-D-galactosidase attached to cells containing antibody to the enzyme. Since the limiting factor in that method appears to be the enzymatic activity present in unimmunized controls, the substitution of AMEF for the enzyme should result in greater sensitivity. At a different degree of sensitivity, using the conventional ONPG assay, the linearity of AMEF activation over the span of seven $\log_2$ units of antibody concentration makes it simpler than most antigen-binding tests.

Summary.—The β-D-galactosidase activity of a protein (AMEF) extracted from a mutant of *E. coli* defective in the *z* gene was increased specifically by anti-β-D-galactosidase serum to a maximum of 550-fold. The purification and biochemical properties of AMEF are described. Evidence was obtained indicating that measurements of antibody by activation of AMEF can reach ultimate levels of sensitivity.

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