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β-Galactosidase: Immunological Activity of Ribosome-Bound, Growing Polypeptide Chains

(immune hemolysis inhibition/antiserum/puromycin/protein conformation/chain folding)

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ABSTRACT **Ribosomes carrying nascent chains of β-galactosidase were prepared by disruption of *Escherichia coli* in detergent-free buffer of high salt concentration, followed by purification on a discontinuous sucrose gradient. Assay by the method of immune hemolysis inhibition with anti-β-galactosidase indicated that considerable amounts of antibody were bound by the growing chains. Much of the crossreacting material could be released from the ribosomes by treatment with puromycin. The ability to bind anti-β-galactosidase was completely destroyed when ribosomes were heated at 60°C. At very early times after induction, well before the appearance of active enzyme, crossreacting material could be demonstrated on ribosomes; this finding correlated with the appearance of an amino-terminal fragment of β-galactosidase. Thus, growing chains of β-galactosidase must begin to fold before their release from the ribosome.**

Under some experimental conditions, nascent peptides longer than 30 or 35 residues are susceptible to attack by proteolytic enzymes (1), a finding that implies that only the growing, carboxyl-terminal portion of nascent peptides is physically shielded from the milieu by the ribosome itself. This observation suggests that as the polypeptide elongates, successively longer portions of the chain will be subject to interaction with solvent, and folding may occur.

It is of considerable interest to determine whether any conformation assumed by a growing polypeptide may contribute to the three-dimensional structure of the finished protein. Studies by Cook and Koshland (2) on the renaturation of several multisubunit enzymes indicate that in the presence of other proteins (such as would obtain in the cell), completely uncoiled polypeptides may find it difficult to reestablish the conformation of the native, multimeric enzyme, suggesting that polymerization would be more efficient if, before release from the ribosome, the individual polypeptides were to assume a conformation capable of accepting the other subunits of the protein. There is some evidence for this view in studies (3–6) in which low levels of an enzymatically-active form of β-galactosidase were demonstrated to be transiently associated with the ribosome. In addition, work on the *in vitro* synthesis of IgG indicates that active antibody molecules may be formed on the ribosome while at least one of the polypeptides of this multisubunit protein is still being synthesized (7).

Several laboratories have examined indirectly the question of conformation of nascent polypeptides. Taniuchi and Anfinsen (8) have demonstrated that after enzymic removal of portions of the carboxyl-terminus of *Staphylococcus* nuclease, these derivatives demonstrate none of the physical or immunological properties of the native molecule, implying that in this small protein the entire amino-terminal sequence is required before any significant degree of folding can occur. Similar observations were reported for sperm-whale myoglobin (9). These results were taken as evidence against folding of nascent polypeptides.

Immunological studies in this laboratory demonstrated that prematurely terminated polypeptides of β-galactosidase produced by nonsense-mutant strains of *Escherichia coli* crossreact with antibodies prepared against the native enzyme (10, 11). Analogous to certain of the derivatives of nuclease and ribonuclease, and to growing polypeptide chains, these nonsense-mutant proteins are missing significant portions of the carboxyl-terminal sequence of β-galactosidase (up to 60% of the entire protein in some cases). The loss of crossreacting ability upon heating or sodium dodecyl sulphate treatment indicates that the antigenicity of these prematurely-terminated polypeptides is conformation dependent.

Here, we test directly whether nascent polypeptides of β-galactosidase, attached through their carboxyl termini to ribosomes, crossreact with antibody prepared against the native enzyme, thus demonstrating conformational similarities to the completed protein. In contrast to other studies in which antibodies were used to demonstrate low levels of presumably *completed* proteins attached to ribosomes, these experiments were designed to measure quantitatively the binding of anti-β-galactosidase to *incomplete* chains.

MATERIALS AND METHODS

Growth of Bacterial Strains and Preparation of Cell Extracts. The wild-type *E. coli* strain, Hfr 3000, the ochre mutant X90, the amber mutant NG 200, and the *lac* deletion strain RV were generously supplied by Dr. Francois Jacob. The "hyper" strain E 203 (12) was obtained from Dr. Aaron Novick and J 90 and M 15 were from Dr. Jonathan R. Beckwith. Cultures were grown with aeration at 37°C in either tryptone broth (13) or in medium 63 (14) containing 1.0% glycerol as carbon source, and were induced with 1 mM β-D-isopropylthiogalactoside (IPTG) (Cyclo Chemical Corp.) for 4 min during the exponential phase of growth, unless otherwise noted. Cells were harvested in an equal volume of ice, washed with ribosome buffer [0.01 M Tris·HCl (pH 7.4)–0.15 M KCl–0.015 M Mg acetate] and the pellet was stored at −40°C. Cells were thawed, suspended in an appropriate small volume of ribosome

Abbreviation: IPTG, isopropyl-β-D-thiogalactoside.

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buffer, and (with the exception of J 90) broken in an Aminco French pressure cell at 18,000 psi. After incubation at 4°C for 15 min with 10 μg/ml of DNase (Worthington Biochemical Corp.), the clear extract was obtained by centrifugation at 27,000 × *g* for 15 min, and usually contained 15–20 mg of protein/ml of extract, as determined by the method of Lowry *et al.* (15).

Strain J 90, which contained an integrated λ-φ80*dlac* region with a temperature-sensitive λ-repressor, was also used. Replication of the defective phage genome was induced by three cycles of temperature shift to 45°C, followed by 10-min incubations with aeration at 40°C. The culture was finally incubated at 37°C for 22 min, and induced with 1 mM IPTG. The cells were harvested by centrifugation with ice, and the pellet was frozen and stored at −40°C. Upon thawing, the cells lysed spontaneously, and the clear extract was obtained as above.

Preparation of Ribosomes. Ribosomes were obtained by layering 2–3 ml of clear extract onto a discontinuous sucrose gradient prepared by layering 5 ml of 30% sucrose over 2.5 ml of 60% sucrose in 10-ml polyallomer tubes. Sucrose solutions were prepared in ribosome buffer. The gradients were centrifuged in a Spinco 40 rotor for 2.3 hr at 39,000 rpm in a Spinco model L265B preparative ultracentrifuge. Fractions were collected and read for absorbance at 260 nm; the ribosomal peak was pooled and dialyzed against ribosome buffer. Sedimentation values were kindly determined (16) by Douglas M. Brown.

Immune Hemolysis Inhibition. The assay for immunological crossreaction is modified from Arquilla *et al.* (17, 18). Indicator cells were prepared by coupling β-galactosidase to the surface of erythrocytes with a diazo compound. Sheep erythrocytes (Davis Laboratories, Inc.) were washed twice with cold, buffered saline [0.01 M sodium phosphate (pH 7.4)–0.15 M NaCl], and 0.1 ml of the viscous pellet was mixed with 1.6 mg of β-galactosidase in 1.5 ml of 0.11 M sodium phosphate (pH 7.4). The mixture was shaken vigorously at room temperature, and 0.35 ml of a freshly diluted solution of the coupling agent bis-diazobenzidine [1:15 in 0.11 M sodium phosphate buffer (pH 7.4)] (18, 19) was added immediately. The cells were mixed for 10 min and washed twice in cold Veronal-buffered saline, and were resuspended in a volume of 0.15% bovine serum albumin in this buffer such that 0.1 ml of the cell suspension in 1.4 ml of H_2O gave an absorbance of 1.2 at 414 nm. Rabbit anti-β-galactosidase was kindly prepared (11) by Dr. Eli Sercarz, and was heated for 30 min at 56°C in order to destroy endogenous complement. Guinea pig complement was purchased as a lyophilized powder from Hyland Laboratories, Los Angeles. It was diluted in Veronal–NaCl plus albumin to a concentration of 50 C'H50 units/ml (20) and stored at −40°C.

Ribosomes (usually 0.1–0.2 mg in 0.1) ml were incubated with 0.1 ml of several different amounts of anti-β-galactosidase for 15 min at 37°C with gentle agitation. At the end of this incubation period, 0.1 ml of β-galactosidase-coated indicator cells and 0.1 ml of the complement solution were added, and the incubation at 37°C was continued for 30 min with vigorous agitation. To each tube was added 1 ml of cold Veronal–NaCl, the tubes were centrifuged at 6000 × *g*, and the adsorbance resulting from cell lysis and hemoglobin release was measured at 414 nm.

RESULTS

Cell Disruption and Purification of Ribosomes. Several standard techniques of cell disruption and ribosome purification were tested. In all cases, concentrated suspensions obtained by these methods were cloudy and difficult to assay in the spectrophotometer, and interfered significantly with several immunoassays. A satisfactory method consisted of cell disruption by the French press in high-salt buffer containing no detergent, followed by purification of ribosomes on a discontinuous sucrose gradient. For the special case of strain J 90, the step involving the French press was omitted. A typical fractionation of the clear extract by this method is shown in Fig. 1. No precautions were taken to prevent degradation of mRNA by endogenous nucleases; 95% of the material in the peak was determined to have a sedimentation value of 70 in the analytical ultracentrifuge.

For wild-type cultures induced for 4 min at 37°C, the associated β-galactosidase enzyme activity of the pooled 70S ribosomal fraction after separation on the gradient was 2–6 units of β-galactosidase per A_{260} unit (66 μg) of ribosomes. This activity is comparable to or lower than that after 4–5 cycles of differential centrifugation (1), and is below the sensitivity of the immunological assay. When strain J 90 was induced for 4 min at 37°C, the β-galactosidase activity was roughly 4-fold higher.

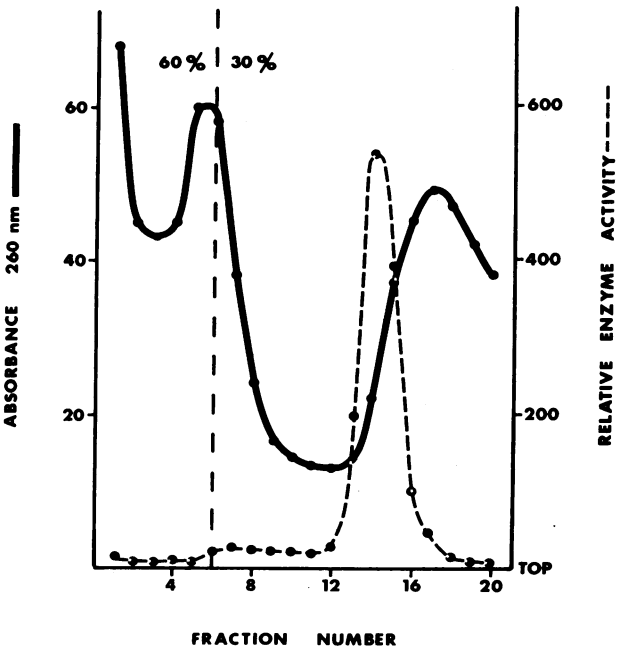

FIG. 1. Purification of ribosomes on a discontinuous sucrose gradient. A clear cell extract obtained from 250 ml of a log-phase culture of *E. coli* strain J 90 was layered onto the gradient and centrifuged as described in the text. Fractions were collected, and the relative enzyme activity and absorbance at 260 nm were determined. The *vertical dashed line* represents the original position of the meniscus between the 60 and 30% sucrose. Fractions 1–6 were pooled and dialyzed against ribosome buffer; any cloudiness was removed by centrifugation at 27,000 × *g* for 15 min. The clarified material was used directly as the source of nascent β-galactosidase polypeptides. In this particular experiment, a considerable amount of heavy polysomal material was recovered, as well as the 70S material in the peak at the 60–30% boundary.

Immune Hemolysis Inhibition. The assay depends upon the quantitation of free antibody remaining after incubation of β-galactosidase or crossreacting material with known, standard concentrations of anti-β-galactosidase. Fig. 2 presents an experiment in which the binding of different quantities of anti-β-galactosidase to three different amounts of β-galactosidase was determined in the presence of β-galactosidase-free ribosomes purified from *E. coli* strain RV, which has a deletion of the entire *lac* region. These curves were virtually identical to the one obtained when the same experiment was done with the ribosomes omitted, indicating that ribosomes do not interfere in the assay.

Binding of Anti-β-Galactosidase to Growing β-Galactosidase. To test whether growing β-galactosidase chains attached to ribosomes bind anti-β-galactosidase, ribosomes were purified from strain J 90, which produces 4- to 5-times more β-galactosidase than a wild-type strain after heat shock and induction. The experiment illustrated in Fig. 3 indicates that ribosomes from J 90 bind a significant amount of anti-β-galactosidase.

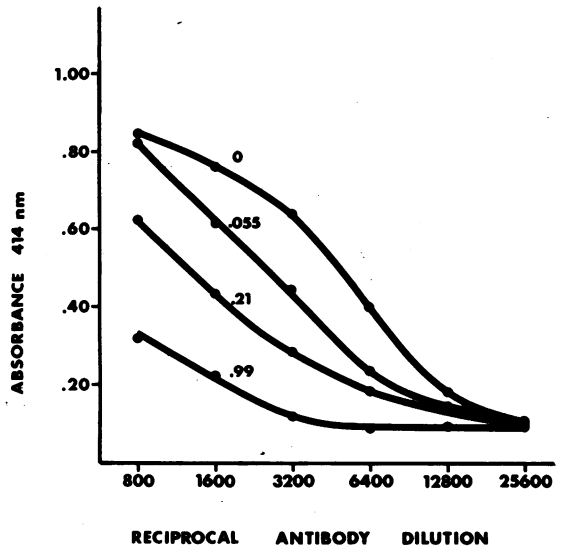

FIG. 2. Estimation of antibody binding to β-galactosidase in the presence of *lac* deletion ribosomes. Three concentrations of β-galactosidase (*GZ*) in the presence of 0.15 mg of *lac*-deletion (*RV*) ribosomes were incubated with several dilutions of anti-β-galactosidase. The free antibody remaining was then measured by its ability to lyse a fixed number of β-galactosidase-coated erythrocytes in the presence of complement. The hemoglobin released upon lysis of the erythrocytes was determined spectrophotometrically at 414 nm, and the absorbance was plotted against the respective antibody dilution. The difference in area between the 100% antibody curve (*lac* deletion ribosomes only) and the curves obtained when the antibody dilutions were first incubated with three concentrations of β-galactosidase is proportional to the amount of antibody bound.

Added GZ in μg	Relative curve area	% Anti-GZ bound
0	100	0
0.055	81	19
0.21	53	47
0.99	21	79

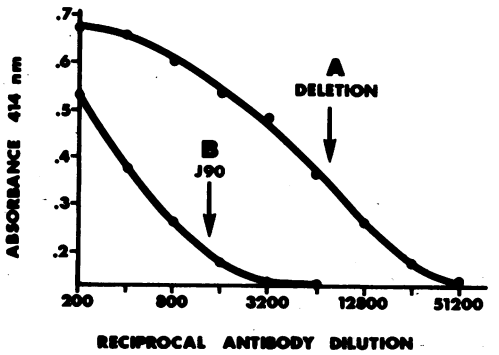

FIG. 3. Binding of anti-β-galactosidase to ribosomes purified from an induced culture of J 90. Ribosomes were purified from an induced culture of J 90 and from the *lac* deletion strain, RV. 0.15 Mg of each type of ribosome was tested for the ability to bind anti-β-galactosidase in the immune hemolysis inhibition assay.

Slightly more than 70% of the antibody was bound by 0.15 mg of ribosomes. The control sample contained the same amount of ribosomes prepared from the deletion strain RV. From Fig. 2, this amount of antibody binding corresponds to 1 μg of β-galactosidase. When the J 90 ribosomes were assayed for associated enzyme activity by the standard *O*-nitrophenyl-β-D-galactoside assay, a value of 0.15 μg of β-galactosidase per 0.15 mg of ribosomes was obtained. Thus, 85% of the crossreactivity detected on J 90 ribosomes was due to nascent polypeptides of β-galactosidase.

Puromycin Release of Crossreacting Material. If the crossreacting material associated with strain J 90 ribosomes was due to nascent β-galactosidase chains attached through tRNA to the ribosome, this material should be released by treatment with puromycin (21). Accordingly, part of an extract prepared from an induced culture of J 90 was incubated with puromycin in order to promote release of peptidyl-tRNA from the ribosomes. The culture was pulse labeled with [^{14}C]leucine before harvest; the release of radioactive material from the ribosomes in a typical experiment is demonstrated in Fig. 4*A*. When tested for their ability to bind anti-β-galactosidase in the immune assay, the puromycin-treated ribosomes had lost roughly 50% of the crossreacting material detectable on untreated ribosomes (Fig. 4*B*). This result further indicated that nascent chains, while in the process of elongation, bind to antibody.

When ribosomes prepared from an induced culture of J 90 were heated to 60°C for 30 min, no crossreacting material could be detected, indicating that the antigenic determinants recognized by anti-β-galactosidase are strictly conformation dependent.

Number of Nascent Chains on Ribosomes. To measure the number of β-galactosidase chains bound to ribosomes, an assay was used that detects a segment of the polypeptide chains located very near the amino terminus. This segment, which is released upon CNBr cleavage, can be measured by its ability to restore β-galactosidase enzyme activity to extracts of M 15, a deletion mutant that produces a protein lacking that region of the polypeptide chain†. Since growing peptide chains are synthesized from the amino to the carboxyl

† Lin, S. & Zabin, I., in preparation.

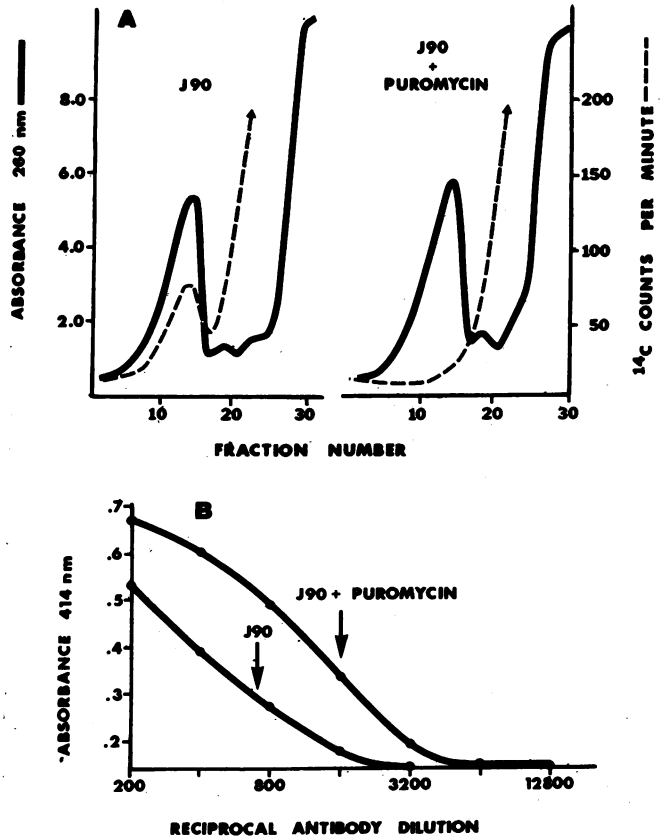

FIG. 4. Puromycin release of crossreacting material from J 90 ribosomes. (*A*) Strain J 90 was induced with IPTG for 4 min and pulsed with 12.5 μCi/ml of [^{14}C]leucine for 20 sec before harvest on ice. An extract was prepared and part of it was incubated for 15 min at 35°C with 250 μg/ml of puromycin and 20 μM GTP. An identical control *without* puromycin was included, and an aliquot of each tube was layered onto linear 5–20% sucrose gradients and centrifuged for 6 hr at 24,000 rpm in a SW25.1 rotor. (*B*) A second aliquot of each sample was applied to the discontinuous sucrose gradient, and the purified ribosomes obtained were tested for crossreacting material by inhibition of immune hemolysis.

end, all chains, except for very short ones, will contain the complementing peptide.

When 1 mg of induced J 90 ribosomes (2.4×10^{14} ribosomes) was treated with CNBr and subsequently assayed for the number of complementing peptides (with β-galactosidase as standard), a value equivalent to 10 μg of β-galactosidase was obtained. Since 10 μg of β-galactosidase corresponds to 4.4×10^{13} monomers, this result implies that $4.4 \times 10^{13}/2.4 \times 10^{14}$ or 18% of the ribosomes carry a nascent β-galactosidase chain.

Kinetics of Appearance of Ribosome-Bound Crossreacting Material. Though unlikely in view of the data presented so far, it can be argued that the crossreacting material associated with ribosomes that is released by puromycin can be largely accounted for by a polymeric form of β-galactosidase, one monomer of which is still in the process of chain elongation. In order to test this possibility, ribosomes from strain E 203 were isolated at very early times after induction and examined for their ability to crossreact with anti-β-galactosidase. E 203 produces high activities of wild-type enzyme upon induction with IPTG, while background levels of enzyme at very early times after induction of J 90 varied considerably. Analyses of enzyme activity, of the number of growing chains, and of antibody binding to E 203 ribosomes are presented in Fig. 5.

Both the number of growing chains, as determined by the CNBr-complementation assay, and the crossreacting material associated with the ribosomes began to increase immediately after induction. This result indicates that the crossreaction associated with the ribosomes at time intervals under 90 sec is due to incomplete, nascent β-galactosidase chains, and not to the presence of highly crossreactive polymeric structures, since the first increase in enzyme activity does not occur until 90 sec after induction. It is also of interest that translation (appearance of the complementing peptide) begins within 20–30 sec after induction. A similar result was obtained by Morrison and Zipser (22).

DISCUSSION

It was demonstrated previously that prematurely-terminated polypeptide chains of β-galactosidase produced by strains containing nonsense codons in the *Z* gene of the *lac* operon crossreact with antibodies prepared against the native, polymeric enzyme (10, 11). Since at least four of these mutant proteins exist in the monomeric state under the conditions of assay (10), it seemed reasonable to assume that many of the antigenic determinants present in the wild-type enzyme are formed in the monomer before polymerization, possibly while the nascent chain is still being elongated. We show that ribosomes carrying nascent chains of β-galactosidase bind antibody prepared against the wild-type enzyme. Most of the crossreacting material associated with ribosomes was released

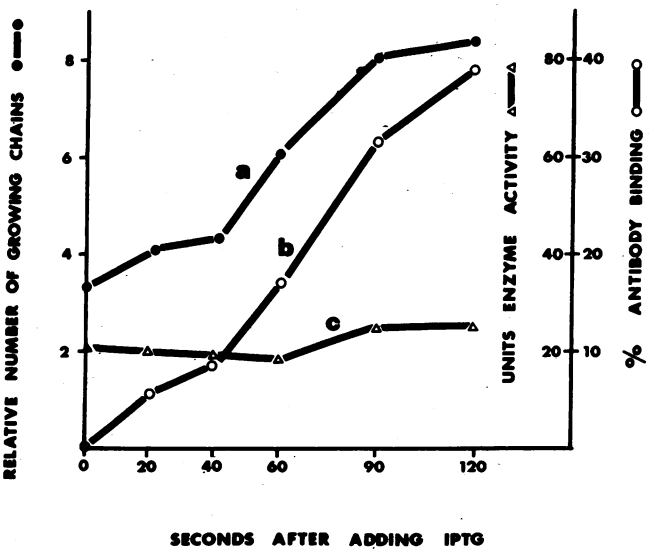

FIG. 5. Rate of appearance of enzyme, growing chains, and crossreacting material in strain E 203. A culture of E 203 was induced with 1 mM IPTG at time zero, and samples were added to an equal volume of ice-cold 0.04 M sodium azide at the times indicated. Extracts were prepared in the French press and the ribosomes were purified. Each ribosomal preparation was assayed for associated enzyme activity (*c*), the number of nascent β-galactosidase chains was determined by the CNBr assay (*a*), and crossreaction with anti-β-galactosidase was measured by inhibition of immune hemolysis (*b*).

by treatment with puromycin. This material could also be detected on ribosomes isolated from cultures at very early times after induction with IPTG, well before the appearance of increased quantities of active enzyme. In addition, all detectable crossreacting material associated with ribosomes from induced cultures was completely destroyed by heating to 60°C. We assume that the ribosome itself has no effect on the folding of nascent chains, and therefore conclude that most of the binding of antibody to ribosomes can be attributed to conformational similarities between nascent β-galactosidase polypeptides and the completed enzyme.

It is possible to compare the relative proportion of antibody bound to nascent chains and to completed β-galactosidase associated with J 90 ribosomes. As determined in the complementation assay, each milligram of ribosomes carries about 4.4×10^{13} nascent chains. By enzyme assay, which detects only native β-galactosidase, each milligram of ribosomes contains 1 μg or 0.4×10^{13} completed chains. Therefore, on a molar basis, the nascent chains comprise 90% of the total. Only 15% of the binding of antibody to J 90 ribosomes could be accounted for by completed, enzymatically-active β-galactosidase; thus, 85% of the antibody binding detected on J 90 ribosomes could be attributed to nascent chains. These values suggest that incomplete, ribosome-bound polypeptide chains bind a considerable quantity of antibody as compared to equivalent molar amounts of completed β-galactosidase.

The results of experiments reported here agree well with earlier work in which enzymatically-active, β-galactosidase-like material was demonstrated to be transiently associated with ribosomes of induced cultures of *E. coli*. It was concluded that a polymeric form of β-galactosidase attached to the ribosome is a normal intermediate in the biosynthesis of the enzyme, since the removal of inducer promoted the release of the fraction (4). The immunological studies presented here demonstrate that this low activity of polymeric, enzymatically-active β-galactosidase accounts for only a minor fraction of the immunological crossreaction observed with anti-β-galactosidase.

The inability of *Staphylococcus* nuclease or sperm-whale myoglobin to fold after removal of a portion of the carboxyl-terminal end of the molecule seems to indicate that small proteins require the entire primary sequence to stabilize the conformation of the protein as a whole (8, 9). In a protein as large as β-galactosidase, there may be several local centers of folding (23), such that the absence of a portion of the polypeptide chain would not disrupt the entire conformation achieved by the completed protein. Experiments reported here and elsewhere support this hypothesis, and suggest that nascent polypeptides begin to assume a conformation that is, by virtue of crossreaction with anti-β-galactosidase, similar to that attained by the completed, functional protein.

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