In order to learn more about the process of intracellular protein degradation, we have attempted to identify in *Escherichia coli* intermediate steps in the rapid degradation of the incomplete β-galactosidase polypeptide that results from the nonsense mutation, X90. The disappearance of this polypeptide (M, approximately 90,000) was followed with gel electrophoresis in sodium dodecyl sulfate, and the auto-α complementation assay was used to detect polypeptides containing the NH₂-terminal end (α region) of β-galactosidase. In addition to the X90 gene product (fragment A), extracts of strain X90 also contain a smaller polypeptide (fragment B) that had a M, if approximately 90,000 and contained the α region. Following removal of inducer and addition of chloramphenicol, total auto-α activity decreased with a half-life of about 30 min. Fragment A disappeared with a half-life of about 6 min. Concomitantly, fragment B increased 2-fold and then decayed with a half-life of about 30 min. The degradation of fragment B appeared to be the rate-limiting step for the disappearance of total auto-α activity from the cell. Fragment B was shown to be an intermediate produced from fragment A by an internal cleavage. Thus, the initial step in the catabolism of abnormal protein seems to be an endopeptidase attack.

Loss of auto-α activity, like degradation of other proteins, requires metabolic energy. When cells were treated with cyanide or azide, degradation of both fragments A and B was blocked. Energy was therefore required for at least two early, sequential steps in protein degradation. The disappearance of fragments A and B was also studied in deg++ mutants which show a reduced ability to degrade nonsense fragments. In these mutants, the level of fragment A was greater, and the relative amount of B much lower than that in deg++ parents. Thus, in the deg++ mutant the conversion of fragment A to fragment B was much slower than in the parent strain.

During degradation or upon treatment with energy inhibitors, no degradative intermediates smaller than B were found by these techniques. Several polypeptides containing auto-α activity were seen on gels, but they appear to be artifacts produced by boiling. When extracts of X90 were treated with mercaptoethanol and sodium dodecyl sulfate and heated to 100° for several minutes, partial cleavage of the polypeptide chain occurred.

In bacterial and animal cells, proteins with highly abnormal structures are degraded much more rapidly than are normal proteins (1, 2). This process serves to rid cells of denatured proteins, mutant polypeptides, proteins resulting from errors in synthesis, or prematurely terminated polypeptides resulting from amber or ochre mutations or from incorporation of puromycin (1, 2). For example, in wild type *Escherichia coli* K12, β-galactosidase is a stable protein, which undergoes no detectable degradation (7, 8). However, a nonsense mutation (X90) in the z-gene leads to production of an incomplete polypeptide which differs in size only slightly from the wild type enzyme, but which is degraded by cells with a half-life of about 7 min (4). The mechanisms for recognition and selective hydrolysis of such abnormal proteins, the nature of the proteolytic process, and the identity of the responsible proteases are not known.

The present studies were undertaken to determine whether the initial cleavage reactions are exopeptidolytic or endopeptidolytic and to define specific steps in the degradative process. We have utilized mutant strains isolated by Bukhari and Zipser (5) that have a reduced ability to degrade nonsense fragments of β-galactosidase. Unfortunately, the nature of the defect in these strains has not yet been determined. One interesting, unexplained feature of the degradation of both normal and abnormal proteins is that this process can be blocked with inhibitors of energy metabolism (2, 9, 10). At first glance, this apparent energy requirement is somewhat surprising since proteolysis must be an exergonic process and since no known protease requires high energy cofactors. These experiments therefore attempted to determine whether high energy compounds are required only for an initial step in protein degradation or perhaps only for the conversion of peptides to amino acids.

In order to investigate these questions, we have attempted to identify intermediates in the intracellular degradation of a single protein, the nonsense fragment of β-galactosidase produced in *E. coli* X90. The characterization of such intermediates should be very useful in defining steps in the degradative
process. Such intermediates, however, might be difficult to detect since they lack enzymatic activity and may be degraded quite rapidly. Since no cell-free system has yet been described which duplicates protein degradation in vivo, these studies were performed with intact cells.

We chose to examine the degradation of a nonsense fragment of β-galactosidase because such inactive polypeptides as well as intermediates in their degradation may be measured by the auto-α complementation assay (11, 12). Morrison and Zipser (11) first showed that when nonsense fragments are autoclaved, an NH₂-terminal polypeptide (“auto-α”; M₀ = 7,400) is released. When auto-α is mixed with an “acceptor” polypeptide containing a deletion within the α region of β-galactosidase, the two inactive polypeptides combine to yield active enzyme (11, 12). This technique permits a quantitative assay for nonsense fragments of β-galactosidase and for any of the degradative products that contain an intact NH₂-terminal region.

Using this assay, Zipser and co-workers (13, 14) found that strain XA21 contained not one, but two, polypeptides containing the NH₂-terminus of β-galactosidase. The larger polypeptide had the expected molecular weight for the mutant polypeptide (M₀ = 90,000). The present studies demonstrate this process.

The amount of ONPG hydrolyzed in this assay was proportional to the amount of auto-α containing extract added and to the time of incubation (data not shown).

**Materials and Methods**

**Strains and Media**—Strains X90 (B1) and XA21 (F' lac i− zA21/i−) were kindly provided by Dr. I. Zabin, University of California, Los Angeles. Lac strain X90 (F pro lac z X90/i pro lac deg T⁺) and X908 (F pro lac z X90/Δ pro lac deg T⁺) were a gift of Dr. Ahmed Bukhari.

LB broth contained 10 g of bacto-tryptone (Difco), 5 g of yeast extract (Difco), and 5 g of NaCl/liter. The minimal medium (15) was supplemented with vitamin B1 (10 μg/ml) and 0.5% glycerol or succinate was added as a carbon source.

**Preparation of Acceptor for Complementation Assays**—Escherichia coli strain XA21 containing a deletion within the α region of the β-galactosidase gene was grown in LB broth at 37°C with aeration and harvested in log phase. All further steps were performed at 0–4°C.

The cell pellet (about 35 g wet weight) was suspended in Buffer I (20 mM Tris-HCl, 5 mM EDTA, 10 mM NaCl, 1% mercaptoethanol, pH 7.2) and washed once by centrifugation for 15 min at 25,000 × g. The cells were suspended by addition of 80 ml of Buffer I and disrupted with a Branson sonifier cell disrupter. After centrifugation for 20 min at 25,000 × g, the supernatant containing auto-α acceptor was decanted. The pellet was suspended by addition of 50 ml of Buffer I, sonicated as before, and centrifuged, and the supernatant was combined with the initial supernatant. After centrifugation for 90 min at 100,000 × g to remove particulate matter, the supernatant (about 50 ml) was removed, and sucrose was added to it to give a final concentration of 10% (w/v). This solution containing acceptor activity was stored in small aliquots at −20°C. Before freezing, acceptor activity was measured with the immunoprecipitation assay using a standard preparation of the method of Morrison et al. (14).

**Degradation Experiments**—Cells were grown at 37°C with aeration for at least three generations in the presence of 5 × 10⁻⁶ M isoprifuothio-β-galactopyranoside. During log phase, cells were collected by pouring over crushed ice and centrifuging for 12 min at 20,000 × g in the cold (4°C). The cells were resuspended in 20 ml of ice cold medium lacking IPTG and centrifuged again at the same speed.

The pellets were suspended in 5 ml of fresh cold medium and dialyzed (at time zero) into 20 to 30 ml of medium at 37°C, and degradation of auto-α activity at 37°C was followed. At various times, samples (1–4 ml) were removed, pipetted onto ice, and centrifuged 15 min at 28,000 × g at 4°C. The supernatants were discarded and the cell pellets were frozen and stored at −30°C. These samples were then either assayed directly for auto-α activity or subjected to gel electrophoresis.

**Auto-α Assay**—Auto-α assays were performed by a modification of the method of Morrison and Zipser (11). Frozen cell pellets were suspended in 0.5 ml of ice-cold Buffer II (20 mM Tris-HCl [pH 7.2], 0.1 M NaCl, 1 mM MgCl₂, 0.5 mM EDTA) in cold tubes and autoclaved for 40 min at 250°C to produce the auto-α polypeptide. After centrifugation to remove the precipitates, supernatants were prepared as auto-α donor in the complementation assay.

To assay auto-α activity, 100 μl of auto-α donor and 50 μl of auto-α acceptor were mixed and incubated at 28°C for 90 min to permit the complementation reaction to occur. (The EDTA in the acceptor preparations aids in its stability but interferes with the assay. Therefore, prior to complementation, MgCl₂ was added to the acceptor solution to give a final concentration of 10 mM.) To assay the complemented enzyme, 0.5 ml of the solution of an auto-α fragment of β-galactopyranosidase was added. The ONPG was dissolved in Buffer II containing 10 mM sodium azide to prevent microbial growth. The enzyme was incubated at 28°C, and when a yellow color had appeared, the reaction was stopped by addition of 0.5 ml of 1 M NaCO₃. Absorbance was read at 420 nm. One unit of enzyme activity equaled that amount which hydrolyzed 1 nmol of ONPG in 1 min at 28°C. Auto-α activity in a sample was defined as the activity of the complemented enzyme formed from that sample. The amount of ONPG hydrolyzed in this assay was proportional to the amount of auto-α containing extract added and to the time of incubation (data not shown).

**SDS Gel Electrophoresis and Determination of Auto-α from Gels**—Gel slabs were prepared according to the methods of Laemmli (16). To prepare samples for gels, frozen cell pellets were dissolved in 0.25 ml of 15% ice cold trichloroacetic acid (TCA) for 24 h with changes of 5-ml aliquots at −30°C. Before freezing, acceptor activity was covered tubes and autoclaved for 40 min at 250°C to produce the auto-α polypeptide. After centrifugation to remove the precipitates, supernatants were prepared as auto-α donor in the complementation assay using a standard preparation of the method of Morrison et al. (14). After electrophoresis, the gel was soaked in 500 ml of 15% ice cold trichloroacetic acid for 24 h with changes of trichloroacetic acid after 2, 5, and 10 h. This treatment fixed the proteins and extracted the SDS. The gel was then neutralized by soaking 15 min with 500 ml of cold buffer (containing 0.04 M Tris base, 0.02 M sodium acetate, 0.002 M sodium EDTA, and brought to pH 10 with NaOH) while shaking gently. The gel was then soaked for 25 min in 250 ml cold Buffer IV (0.4 M Tris base, 0.2 M sodium acetate, 0.02 M sodium EDTA, brought to pH 7.5 with glacial acetic acid), for 10 min in 250 ml of fresh cold Buffer IV, and for 5 min in 250 ml of fresh cold Buffer IV which had been diluted 10-fold with distilled water. After neutralization, the slab gel was examined under black light illumination which caused the dansylated markers to fluoresce. To separate the reticuloendothelial elements, the gel was examined under black light illumination which caused the dansylated markers to fluoresce. To separate the reticuloendothelial elements, the gel was analyzed for fragments of protein, and the fluorescent products were extracted with a strong acid or base according to the method of Laemmli (16).

After electrophoresis, the gel was soaked in 500 ml of 15% ice cold trichloroacetic acid for 24 h with changes of trichloroacetic acid after 2, 5, and 10 h. This treatment fixed the proteins and extracted the SDS. The gel was then neutralized by soaking 15 min with 500 ml of cold buffer (containing 0.04 M Tris base, 0.02 M sodium acetate, 0.002 M sodium EDTA, and brought to pH 10 with NaOH) while shaking gently. The gel was then soaked for 25 min in 250 ml cold Buffer IV (0.4 M Tris base, 0.2 M sodium acetate, 0.02 M sodium EDTA, brought to pH 7.5 with glacial acetic acid), for 10 min in 250 ml of fresh cold Buffer IV, and for 5 min in 250 ml of fresh cold Buffer IV which had been diluted 10-fold with distilled water. After neutralization, the slab gel was examined under black light illumination which caused the dansylated markers to fluoresce. To separate the reticuloendothelial elements, the gel was analyzed by autoradiography of the various samples. The DNA was assayed by a modification of the method of Morrison et al. (14). After electrophoresis, the gel was soaked in 500 ml of 15% ice cold trichloroacetic acid for 24 h with changes of trichloroacetic acid after 2, 5, and 10 h. This treatment fixed the proteins and extracted the SDS. The gel was then neutralized by soaking 15 min with 500 ml of cold buffer (containing 0.04 M Tris base, 0.02 M sodium acetate, 0.002 M sodium EDTA, and brought to pH 10 with NaOH) while shaking gently. The gel was then soaked for 25 min in 250 ml cold Buffer IV (0.4 M Tris base, 0.2 M sodium acetate, 0.02 M sodium EDTA, brought to pH 7.5 with glacial acetic acid), for 10 min in 250 ml of fresh cold Buffer IV, and for 5 min in 250 ml of fresh cold Buffer IV which had been diluted 10-fold with distilled water. After neutralization, the slab gel was examined under black light illumination which caused the dansylated markers to fluoresce. To separate the reticuloendothelial elements, the gel was analyzed for fragments of protein, and the fluorescent products were extracted with a strong acid or base according to the method of Laemmli (16).

**RESULTS**

Initial experiments confirmed the rapid degradation of auto-α-containing polypeptides in Escherichia coli strain X90, and demonstrated the existence in this strain of two protein fragments of β-galactosidase. Cells were grown on glycerol minimal medium containing IPTG, washed to remove inducer, ransoside; dansyl, 5-dimethylaminonaphthalene-1-sulfonfyl.
and incubated in fresh medium. The degradation of auto-\(\alpha\) activity was followed in the presence and absence of chloramphenicol (Fig. 1). Auto-\(\alpha\) activity disappeared with a half-life of 33 min, in agreement with the data of Lin and Zabin (3). No effect of chloramphenicol was noted. The half-life of auto-\(\alpha\) activity depended on the medium in which the cells were grown. In a series of experiments, the half-life of auto-\(\alpha\) activity in X90 was 23 min for cells grown on LB broth, 33 min for cells grown on minimal medium with glycerol, and 80 min for cells grown on minimal medium with succinate.

When extracts from such cells were analyzed by gel electrophoresis in the presence of sodium dodecyl sulfate, polypeptides of two different sizes containing auto-\(\alpha\) activity were seen (Fig. 2A, top) in accord with the observations of Morrison and co-workers (13, 14). The larger polypeptide, designated fragment A, migrated on gels at the same rate as \(\beta\)-galactosidase. The smaller polypeptide, designated fragment B, had a \(M_r = 90,000\) and migrated slightly faster than phosphorylase \(\alpha\). No other auto-\(\alpha\)-containing polypeptides were detected on the gels.

Further experiments attempted to determine whether the smaller polypeptide was produced by a degradation of the larger one or whether it arose by some other mechanism (e.g. premature termination during protein synthesis or translation of an incomplete mRNA). Samples of cells were subjected to electrophoresis at different times after removal of inducer (Fig. 2A). By measuring auto-\(\alpha\) activities in each gel slice, it was possible to determine the total amounts of fragments A and B at various times and to determine the kinetics of disappearance of these fragments (Fig. 2B). (With experience, recovery of auto-\(\alpha\) off gels was reproducible and linear. In one experiment, the sum of activities of fragments A and B off gels gave the same degradative rate as total auto-\(\alpha\) activity, over a 10-fold range.)

Fragment A decayed with a half-life of about 5 min (Fig. 2B), which is consistent with the value of 7 min obtained by Goldschmidt using autoradiographic techniques (4). At the same time, the amount of fragment B increased to twice its initial level, and then disappeared with a half-life of about 30 min. Thus, the decay of fragment B seemed to be the rate-limiting step in the disappearance of auto-\(\alpha\) activity from the strain X90. Data from Fig. 2A and from gels of similar samples taken at other times were used to measure the amounts of fragments A and B (i.e. the total auto-\(\alpha\) activity under each peak). In this experiment a small lag was observed prior to the disappearance of total auto-\(\alpha\) activity (i.e. the sum of A and B). This result was not observed in other experiments of this type (e.g. Fig. 9).
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Fig. 3. Degradation of auto-α activity in strain X90. Cells were grown in LB broth containing IPTG, washed, and resuspended in minimal medium containing 0.5% succinate and chloramphenicol. A, samples were removed at various times and total auto-α activity cell (Fig. 1). In accord with this conclusion, no other intermediates with auto-α activity were detected. The doubling of the level of fragment B in the presence of chloramphenicol could be accounted for only by the proteolytic conversion of A into B. This conversion of A to B appears to be endoproteolytic since polypeptides containing auto-α activity were never found on the gels between peaks A and B. Furthermore, during degradation, peaks A and B decreased in height without changing in width. A similar observation was made for peak A by Goldschmidt (4) using autoradiography.

When auto-α assays were performed for longer incubation times, several smaller peaks of auto-α activity were evident (Fig. 3B). These results raised the possibility that other degradative intermediates might also be present in strain X90. However, these additional peaks appear to be experimental artifacts produced by heating the samples in SDS sample buffer prior to electrophoresis. Table I illustrates that the amount of auto-α activity with mobility greater (i.e., smaller molecular weight) than that of fragment B increased with the time of heating the samples. If the sample was not boiled, but was incubated for 40 min at 60°C, only 5% of auto-α activity was found in this region. Samples boiled for 5 or 20 min contained 27 and 53%, respectively, of this auto-α activity in the lower molecular weight region of the gel. For most subsequent experiments, samples for SDS-gel electrophoresis were boiled for only 1 min, which led to 17% of the auto-α activity being in the lower M region of the gel. However, the ratio of the activity in fragment A to the activity in fragments A + B was fairly constant and independent of the time of boiling. Thus, on heating, material was lost from both of these polypeptides proportionally, and fragment A was not converted to fragment B by the boiling procedure.

When cells were permitted to degrade auto-α activity for long periods of time, as in the experiment of Fig. 3, the degradation rate decreased. For example, in this experiment, the half-life for auto-α activity was 25 to 30 min during the 1st hour. During the remaining 1½ h, the half-life was 150 min (Fig. 3A). Similar kinetics have been observed by previous workers (3). This unexplained decrease in the rate of degradation was not due to accumulation of a stable degradative intermediate since, even at 150 min, the residual auto-α activity was found in fragments A and B (Fig. 3B).

Energy Requirement for Protein Breakdown – The identification of an intermediate step in protein catabolism allowed us to investigate whether metabolic energy was required at a prior or a subsequent step. Induced cells were washed and suspended in minimal medium containing succinate, chloramphenicol, and azide, or in minimal medium containing cyanide. In cells growing on succinate, energy can be derived only from aerobic respiration. As shown in Fig. 4, both cyanide and azide blocked degradation of auto-α activity almost completely. This effect of cyanide was reversible, and did not involve a change in cell viability. Removal of cyanide resulted in rapid degradation (data not shown). Thus, the degradation of incomplete β-galactosidase molecules is an energy-requiring process, as has also been observed by Zipser and co-workers (3). By analyzing the levels of fragments A and B following energy inhibition, it was possible to determine whether metabolic energy was required for the initial conversion of A to B, for a later step, or throughout the degradative pathway.

The effects of cyanide on the levels of fragments A and B were analyzed by electrophoresis (Fig. 5) using the same cells studied in Fig. 4. In the absence of cyanide, fragment A decayed with a half-life of about 17 min or less (Fig. 5). The level of fragment B remained high for 30 min, presumably due to the continued conversion of A to B, and then decreased. Treatment with cyanide, however, markedly inhibited the

| Treatment       | Auto-α activity smaller than fragment B (% of total auto-α) | Activity in peak A activity in A and B |
|-----------------|------------------------------------------------------------|---------------------------------------|
| 40 min at 60°C  | 8                                                          | 0.56                                  |
| 1 min at 100°C  | 17                                                         | 0.64                                  |
| 5 min at 100°C  | 27                                                         | 0.56                                  |
| 20 min at 100°C | 52                                                         | 0.56                                  |
| 20 min at 100°C (duplicate) | 55                                           | 0.57                                  |
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Fig. 4. Effect of energy inhibitors on degradation of auto-α activity in strain X90. Left, cells were grown in minimal medium containing 0.5% glycerol, 0.1% casamino acids (Difco), and IPTG. Cells were washed and resuspended in minimal medium containing chloramphenicol. One-half of the cells also were treated with 1.4 mM KCN. Right, cells were grown in LB containing IPTG, washed, and resuspended in minimal medium containing 0.5% succinate and chloramphenicol. One-half of the cells also were treated with 25 mM sodium azide.

Fig. 5. Effect of cyanide on degradation of fragments A and B in strain X90. Cells from the experiment of Fig. 4 were used to analyze the degradation of the individual fragments in the presence and absence of cyanide. For each time, a sample from the control and a sample from the cyanide-treated cells were electrophoresed on SDS gels. The levels of A and B at each time were determined from the total auto-α activity under each peak (A and B). For the control, duplicate gels were prepared at 0 and 90 min and data from both have been included.

disappearance of both fragments A and B. It is also noteworthy that during cyanide treatment no new fragments accumulated with time (data not shown). Thus, energy is required for at least two sequential steps in the degradation of this protein, the conversion of A to B and the subsequent hydrolysis of B.

Treatment with azide had effects similar to those of cyanide. Azide completely blocked degradation of total auto-α activity in the cells (Fig. 4), inhibited the loss of the fragments A and B, and did not cause the appearance of any new peaks (Fig. 6). However, when measured from the gels, about 45% of both peaks A and B disappeared in 2½ h. Thus, after azide treatment (but not after cyanide) some of the activity present in cells was not detected in the gels. Several explanations may account for this discrepancy. A and B might be partially cleaved to acid-soluble polypeptides that contain intact α regions and that may leach out of the gels during the extensive soaking in trichloroacetic acid. However, during azide treatment, no acid-soluble auto-α activity was detected, and acid-precipitable auto-α activity remained constant (data not shown). Alternatively, A and B may have been converted into a form which did not enter the acrylamide gels; for example, clumping of cells occurred upon treatment with azide, but not with cyanide; also during azide treatment the abnormal polypeptides may have associated into dense intracellular aggregates (10, 18). This latter possibility is now being investigated.

Studies of Deg- Mutants—The deg- mutants (5) degrade nonsense fragments of β-galactosidase at reduced rates. To test whether the mutations affect the degradation process, we have analyzed the degradation of polypeptide fragments A and B in such mutants. Induced cells of deg+ and deg- carrying the X90 mutation were washed and suspended in inducer-free medium with chloramphenicol and degradation was followed (Fig. 7). The half-lives were 280 min for deg+ and 71 min for deg-. Cell samples from this experiment were analyzed on gels in order to follow the fates of individual fragments. At time zero (Fig. 8), the deg- strain contained fragments A and B in approximately a 1:1 ratio. Cell samples from this experiment were analyzed on gels in order to follow the fates of individual fragments. At time zero (Fig. 8), the deg+ strain contained fragments A and B in approximately a 1:1 ratio. Samples taken at various times resolved this question (Fig. 9). In the deg+ parent, fragment A decreased with a half-life of 8 min while B at first rose by 30% of its original value (due to continuous conversion of A to B) and then fell. In the mutant, however, the rate of decay of fragment A was greatly reduced. From these data, it was not possible to determine whether the degradation of fragment B was also inhibited even though the level of B decreased more slowly in deg- . Since A remained higher than B during the
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Fig. 7. Degradation of incomplete polypeptides in strains deg+ and deg−. Cells of strain 8057 (deg+) and 8058 (deg−) carrying the X90 mutation were grown on LB broth with inducer, then washed and incubated in fresh medium containing chloramphenicol (t = 0). Samples were removed at various times after resuspension to determine total auto-α activity.

Fig. 8. Distribution of fragments A and B in strains deg+ and deg−. Cells from the experiment of Fig. 7 were removed at t = 0 and analyzed for auto-α activity following SDS gel electrophoresis. No other peaks of activity were found on these gels.

Fig. 9. Degradation of fragments A and B in strains deg+ and deg−. Cells from the experiment of Fig. 7 were analyzed for the levels of fragments A and B at various times following removal of inducer. At each time, a sample from the deg+ and a sample from the deg− cultures were electrophoresed. The levels of A and B at that time were determined in the usual fashion.

DISCUSSION

These experiments have demonstrated that the rapid degradation of the abnormal protein resulting from the nonsense mutation X90 involves cleavage to a more stable polypeptide. A precursor-product relationship was established between the initial gene product, fragment A (M, = approximately 120,000) and fragment B (M, = 90,000). Thus in the absence of protein synthesis, the level of B increased up to 2-fold as fragment A decreased (Figs. 2B and 9A). Fragment B is one of the first intermediates in the degradation of intracellular proteins to be identified. No other intermediates were demonstrable with the auto-α assay. Thus, the disappearance of fragment B may involve cleavage within the α region. Alternatively, if other intermediates containing the NH2-terminal region exist, they must have been hydrolyzed very rapidly and consequently were not detected.

Our studies support the suggestion first made by Goldschmidt (4, 13) that fragment R is derived from A by an endoproteolytic cleavage. No polypeptides intermediate in molecular weight between fragments A and B were seen at any time (Fig. 2A). If an exoprotease were responsible for the conversion of A to B, auto-α activity should be present between the two fragments. On gel electrophoresis, A and B were sharp peaks. Furthermore, their width did not increase with time as the peak heights decreased. This result is in agreement with an endoproteolytic, rather than exoproteolytic cleavage. It is noteworthy that the deg- mutation inhibited the A to B conversion.

In the deg- cells, the absolute level of fragment A was increased and the proportion of auto-α activity in fragment B was greatly reduced (Fig. 8) as a result of the slower degradation of fragment A (Fig. 9). Similar results were also obtained with independently isolated deg- mutants (deg R− and deg T−). Recent genetic studies suggest that these separately isolated mutants may in fact be within the same cistron. Additional evidence that the deg- mutation blocks an endoproteolytic cleavage of another abnormal polypeptide was obtained by Villarejo and Zabin (19). Real proof of an endoproteolytic mechanism would involve the identification of the

K. Murakami, Y. Klemes, and A. L. Goldberg, unpublished observations.

5 S. Gottesman, personal communication.
carboxyl fragment resulting from the cleavage. Since this molecule should not be detectable by the auto-\(\alpha\) assay, we have attempted to find it using antibodies against \(\beta\)-galactosidase. These antibody experiments showed similar proportions of peak A and B and similar kinetics of decay as were obtained by assaying \(\alpha\)-complementing activity. Although they confirmed the precursor-product relationship between A and B, they have as yet failed to detect a carboxyl fragment. (In the absence of such data, we cannot completely eliminate the possibility that an exoproteinase removes amino acids very rapidly from the carboxyl end of fragment A, until fragment B remains.)

It has generally been assumed that an initial proteolytic cleavage is the rate-limiting step in the degradation of a protein to amino acids. However, the half-life of fragment B (about 30 min) was at least 5 times longer than that of A (Fig. 2B). Thus, decay of fragment B seems to be the rate-limiting step for disappearance of auto-\(\alpha\) containing polypeptides from the cell. Several other nonsense mutants have been found to contain two polypeptide fragments of \(\beta\)-galactosidase, one corresponding in size to the presumed gene product and the other smaller (13, 14). Fragment B should correspond to the map position 11 of Lin and Zabin (3) which corresponds to a \(M_c \approx 60,000\), as is fragment A (Figs. 5 and 9A) and the half-lives of auto-\(\alpha\) activity in these cells approximate that of fragment B. For those mutants in which the nonsense fragments should be smaller than B, the half-lives are much shorter with one exception (3). These observations suggest that in strains with nonsense fragments greater than \(M_c \approx 90,000\), an initial endoproteolytic cleavage always occurs at the same site to yield fragment B, plus a peptide whose size varies with the mutant. Fragment B is relatively stable compared to smaller auto-\(\alpha\) containing polypeptides, whose half-lives may be as short as a few minutes (3). Thus the half-life of B should be rate-limiting for decay of auto-\(\alpha\) activity in all strains with large nonsense fragments (map position 11 or greater).

These studies have failed to demonstrate any other smaller degradative intermediates in X90. Goldschmidt reported a \(M_c \approx 60,000\) polypeptide containing auto-\(\alpha\) activity on SDS gels that represented 6% of the total auto-\(\alpha\) activity in strain X90 (13). We have also found this peak and several additional smaller ones (Fig. 3B). However, the amount of auto-\(\alpha\) activity in polypeptides with \(M_c < 90,000\) depended on the method of treatment of the samples. The more gentle the treatment prior to electrophoresis, the less activity was found in these low \(M_c\) peaks (Table I). Thus, these polypeptides appeared to result from cleavage of both fragments A and B induced by boiling. To our knowledge, there are no previous reports of hydrolysis of proteins upon boiling in SDS (20). However, the formation of the auto-\(\alpha\) polypeptide is by itself evidence that high temperatures can cause proteolytic cleavages at specific sites. In addition, boiling of commercially obtained pure \(\beta\)-galactosidase causes a decrease in the molecular weight of the polypeptide. It is also possible that the small fragments (Table I) represent degradative intermediates which are tightly, but not covalently, bound to the complementary polypeptide, so that SDS treatment does not dissociate them without heating. It is known that \(\beta\)-galactosidase can be cleaved in many places, and still retain its tertiary structure and enzymatic activity (21). However, there is no evidence that such strong binding may occur in the presence of SDS.

The term "half-life" has been used a bit loosely in this paper and by others (3, 5) to describe the decay of a heterogeneous population of polypeptides all containing auto-\(\alpha\) activity (e.g., Fig. 1) even though this process is in fact not strictly exponential. Lin and Zabin (3) first noted that the rate of decay of auto-\(\alpha\) activity in strains carrying X90 or other nonsense mutations was not exponential, but decreased after a period of time which varied in different mutants. This finding has been confirmed in the present studies (Fig. 3A) which further show that this decrease in auto-\(\alpha\) decay is not due to the accumulation of new stable polypeptides containing the auto-\(\alpha\) region (Fig. 3B). Residual activity was found principally in fragments A and B. The degradation of fragments A (Figs. 5 and 9A) and B (data not shown) was also initially rapid but decreased markedly when these polypeptides were present in very low amounts.

It is interesting that these biphasic kinetics were obtained only in \(deg^+\) cells. In \(deg^-\) strains, the initial rapid decay process appears to be lacking (Fig. 7). Possibly, these results suggest the existence of two proteolytic systems capable of attacking nonsense fragments, one responsible for the rapid degradation of auto-\(\alpha\) polypeptides, that is defective in \(deg^-\) cells, and one for the later, slower degradative process apparent in \(deg^-\) cells only with low levels of substrate. This laboratory has made extensive efforts to find differences between the proteolytic activity of extracts of \(deg^+\) and \(deg^-\) strains without success (22).

The degradation of the auto-\(\alpha\) containing polypeptides, like other cell proteins (9, 10), appears to require metabolic energy (Fig. 4). This process was blocked almost completely by both cyanide and azide which inhibit respiration. In addition, cyanide (Fig. 5) and azide (data not shown) reduced both the conversion of A to B and the subsequent hydrolysis of fragment B. Therefore, energy is required for at least two and possibly more steps in the degradative pathway. Related studies in this laboratory indicate that energy is also required for breakdown of much smaller fragments of \(\beta\)-galactosidase (\(M_c \approx 8,000\)) (22). It has been suggested that metabolic energy is required for compartmentalization of proteins in some degradative structures (e.g., periplasm or lysosome) or to activate the protease or for chemical modification of the proteins to make them more susceptible for degradation (2). In either case, the finding that energy is required for multiple steps in degradation would suggest that repeated modification of the substrate is necessary or that the protein must undergo repeated uptake into such a degradative compartment.

The pathway of protein degradation probably can only be elucidated with cell-free preparations. Until very recently, it has proved impossible to demonstrate energy-dependent proteolysis with cell-free extracts. However, recent experiments from this laboratory have demonstrated ATP-dependent degradation of abnormal proteins (including certain low \(M_c\), nonsense fragments of \(\beta\)-galactosidase) in extracts of E. coli (22) and rabbit reticulocytes (23). Further studies with these cell-free systems should help clarify the precise role of ATP in the degradative process. Toward this goal, the identification of an intermediate in the degradative process should prove of value not only in defining a specific proteolytic step, but also in providing criteria for evaluating whether such cell-free preparations reproduce the in vivo process.

\* J. D. Kowit and Y. Klemes, and A. L. Goldberg, unpublished observations.

\* J. D. Kowit and J. Maloney, unpublished observations.
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