Correlation Between Rates of Degradation of Bacterial Proteins In Vivo and Their Sensitivity to Proteases
(protein conformation/abnormal proteins/aminoc acid analogs/mistranslation)

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ABSTRACT
Experiments were undertaken to test whether the relative rates of degradation of proteins in vivo might correlate with their sensitivity to proteases. Various experimental conditions that promote degradation of proteins in Escherichia coli increased sensitivity of average cell proteins to trypsin or other endoproteases, including incorporation of several amino acid analogs into the proteins, incorporation of puromycin into the polypeptide chain, or frequent errors in translation. Those abnormal proteins that were degraded most rapidly within the cell appeared responsible for the increased protease sensitivity of the cell extract. Normal E. coli proteins that rapidly turn over in growing cells are, on the average, more sensitive to trypsin or Pronase than cell proteins that turn over more slowly. The inherent sensitivity of a protein to proteolytic digestion is thus a major determinant of protein half-lives in vivo.

Various proteins within mammalian (1, 2) or bacterial (3) cells differ appreciably in their stability. In rat liver, for example, the half-life of the average protein is about 3 days, while the half-lives of individual enzymes range between 11 min and many days (1, 2). It appears likely that these different rates of protein degradation in vivo reflect inherent differences in protein conformations. Recently several groups have demonstrated in Escherichia coli that alterations in the primary structure of a protein can affect its rate of catabolism (4-7). For example, in E. coli a mutant lac repressor with an altered carboxyl terminal (6) and certain nonsense fragments of z-galactosidase (7) are both rapidly catabolized even though the wild-type proteins are stable. In addition, the incorporation of puromycin (4, 5) or various amino acid analogs into proteins leads to the rapid hydrolysis of the abnormal proteins (4, 5, 8). Finally, increased mistranslation of the genetic message (e.g., in bacterial strains carrying either a ribosomal mutation or nonsense suppressor) is also associated with an increased rate of protein breakdown (4).

The manner in which such alterations in the primary sequence of proteins affect their half-lives remains to be elucidated. The simplest possibility is that in the altered conformation a protein becomes more sensitive to proteolytic enzymes normally found in the cell. The present studies were undertaken to determine whether a general correlation might exist between the rate of protein catabolism in vivo and the sensitivity of cell proteins to proteolytic digestion. We demonstrate that the various conditions that promote protein breakdown in E. coli also increase the sensitivity of cell proteins to attack by pancreatic proteases in vitro. In addition, we have found that those normal proteins that turn over rapidly in growing E. coli appear inherently more sensitive to proteases than proteins that turn over more slowly.

METHODS AND MATERIALS
E. coli auxotrophs were grown on glucose-minimal medium supplemented with required amino acids for two generations, as described (4). For the study of the effects of incorporation of amino acid analogs, growing cells were centrifuged, washed, and suspended for 12 min in the presence of either the required amino acid or equimolar concentrations of the appropriate analogs (4) before receiving radioactive leucine. To obtain polypeptides containing puromycin (4, 5), I added puromycin to growth medium for 12 min. [3H]Leucine (2 μCi/ml, 50 Ci/mmol, Schwartz/Mann Biochemical Co.) was then added to such cultures or to normally growing controls for 5 min to label cell proteins. The cells were then filtered, washed, and suspended in the original growth medium, supplemented with 75 μg/ml of leucine to prevent reincorporation of [3H]leucine.

For measurements of protein breakdown in vivo, the labeled cells were allowed to grow in the presence of the required amino acids, and proteolysis was estimated from the release of [3H]leucine into Cl4COCOOH-soluble form (4). For the study of the protease sensitivity of cell proteins, the labeled cells were centrifuged at 4° and lysed by freezing and thawing in the presence of lysozyme (9). The disrupted cells were incubated at 37°C in cold buffer containing 50 mM Tris-HCl (pH 7.5)–10 mM magnesium sulfate–0.2 M KCl. The supernatants were treated with proteases immediately after centrifugation, since repeated freezing and thawing appeared to increase protease sensitivity.

Extracts were incubated with trypsin (Boehringer–Soehne), chymotrypsin (Sigma, Type II), Pronase (Worthington), or subtilisin (Sigma) in the buffer. The actual concentration of the enzyme used varied in the different experiments, but in each case the proteases were present in sufficiently high concentration that the rate of protein hydrolysis was much faster than the rate found in the intact cells, and that changes in extract concentration did not affect apparent protease sensitivity. Percentage of protein breakdown was expressed as the amount of trichloroacetic acid-soluble radioactivity relative to the amount initially found in proteins.

RESULTS
Incorporation of various amino acid analogs into cell proteins increases their rate of degradation in vivo (4, 5, 8). To compare the protease sensitivity of proteins containing analogs with normal proteins, E. coli auxotrophs were allowed to
TABLE 1. Effects of different proteases on proteins containing aminoacid analogs or puromycin

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Proteins synthesized in presence of</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Subtilisin</th>
<th>Pronase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mg/ml)</td>
<td></td>
<td></td>
<td>(mg/ml)</td>
</tr>
<tr>
<td>1</td>
<td>Arginine (20)</td>
<td>72</td>
<td>43</td>
<td>56</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Canavanine (20)</td>
<td>92</td>
<td>58</td>
<td>73</td>
<td>89</td>
</tr>
<tr>
<td>2</td>
<td>Tryptophan (60)</td>
<td>49</td>
<td>51</td>
<td>62</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>7-Azatryptophan (60)</td>
<td>65</td>
<td>58</td>
<td>76</td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td>Required amino acids</td>
<td>49</td>
<td>51</td>
<td>62</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>+ Puromycin (300)</td>
<td>61</td>
<td>62</td>
<td>80</td>
<td>68</td>
</tr>
</tbody>
</table>

Extracts of E. coli A33 were incubated for 45 min in the presence of 20 μg/ml trypsin, 20 μg/ml subtilisin, 20 μg/ml Pronase, or 40 μg/ml chymotrypsin. In all experiments, the cells were exposed to [3H]leucine in the presence of the indicated amounts of amino acid or analog. Experiment 1: Extracts containing canavanine contained 0.61 mg/ml of protein; the control, 0.46 mg/ml. Experiment 2: extracts containing 7-azatryptophan contained 0.50 mg/ml of protein, the control, 0.46 mg/ml. Experiment 3: the puromycin-treated extract contained 0.46 mg/ml protein; the control, 0.50 mg/ml.

incorporate [3H]leucine in the presence of analogs or required amino acids (4). Extracts from such cells were then incubated with proteolytic enzymes. Fig. 1 compares the trypsin sensitivity of cell proteins synthesized in the presence of arginine or canavanine, proline or azetidine carboxylic acid, and valine or 2,3-threo-α-amino-2-chlorobutyric acid. In each case, proteins containing the analog were hydrolyzed more rapidly than the normal proteins. Differences in protease sensitivity were observed repeatedly, although the magnitude of the differences varied in individual experiments and with different incubation conditions. In other experiments, lower amounts of trypsin were found to produce greater differences in rates of hydrolysis of the cell extracts than those reported here. However, the present experiments used high protease concentrations in order to insure that the rate of protein hydrolysis in cell extracts greatly exceeded that found in intact cells and thus to avoid possible complications in interpretation (see below). Analogous findings were obtained in experiments with other analogs whose incorporation into cell proteins also promotes protein catabolism (4), such as azatryptophan (Table 1) or 5-fluorotryptophan in place of tryptophan, fluoro tyrosine in place of tyrosine, and (β-aminoethyl) cysteine in place of lysine. The observed differences (Fig. 1) in protease sensitivity must result from incorporation of the analogs into the labeled proteins, since addition of canavanine or azetidine carboxylic acid to control cells after exposure to [3H]leucine did not alter the trypsin-sensitivity of the preformed (labeled) proteins. In addition, when an arginine auxotroph incorporated both [14C]canavanine and [3H]arginine, the proteins containing the analog, which are degraded more rapidly in vivo, were also found to be more sensitive to trypsin in vitro (unpublished observations).

In addition to proteins containing aminoacid analogs, E. coli selectively degrade incomplete polypeptides, which had incorporated puromycin and were prematurely released from the ribosome (4, 5). Fig. 1D demonstrates that polypeptides made in the presence of puromycin are also more sensitive to trypsin than normal cell proteins. In addition,
protein breakdown occurs more rapidly in strains that produce frequent errors in translation, such as those carrying ribosomal ambiguity mutations (ram), than in control cells (4). Fig. 2 indicates that the average proteins made in a ram 1 strain are more sensitive to tryptic digestion than average proteins from an isogenic wild-type strain. Similar observations have also been made in comparisons of the trypsin-sensitivity of isogenic ram 2 and ram + strains.

These data suggest a general correlation between rates of intracellular catabolism and sensitivity to trypsin. Analogous results were also obtained with other endoproteases of different specificities. As shown in Table 1, proteins containing canavanine or azatryptophan were more sensitive to chymotrypsin, subtilisin, and Pronase than those containing normal amino acids. Similarly, puromycin-containing proteins were more sensitive to Pronase and chymotrypsin than average cell proteins (Table 1).

Experiments were also undertaken to determine whether the abnormal proteins that are rapidly degraded by cells are the ones responsible for the increased protease-sensitivity observed in vitro. After exposure to canavanine and [3H]leucine, half of the culture was frozen. The other half was permitted to grow in medium containing arginine and non-radioactive leucine. After one or two generations (1 or 2 hr), these cells were frozen. By this time, they had degraded 48% and 78% of the labeled analog-containing proteins, respectively. Concomitantly, these cells appeared to have lost their most easily digested proteins since the remaining radioactive proteins were significantly less sensitive to the protease than the proteins of the cells frozen at earlier times (Fig. 3). In fact, by 2 hr after exposure to canavanine, the intracellular rates of protein breakdown had decreased to levels similar to those seen in control cells exposed only to arginine. At this time the remaining radioactive proteins in the extracts did not differ in trypsin sensitivity from labeled proteins of control cells. Thus there was a very strong correlation between rates of protein degradation in vitro and trypsin sensitivity in vitro.

Normal proteins

Normal cell proteins in E. coli also vary appreciably in their rates of degradation (3), and experiments were undertaken to determine whether such differences in protein half-lives can also be explained by differences in sensitivity to protease. Growing cells received [3H]leucine for 5 min. The cells were then either frozen or permitted to grow in the presence of non-radioactive leucine for 2 hr. Immediately after the radioactive pulse, the apparent rate of degradation of labeled proteins exceeded that observed at the later times (i.e., the measured rate of proteolysis decreased as the cells lost their more labile components). The radioactive proteins remaining in the cells at later time were found to be less sensitive to trypsin than those synthesized 1 hr earlier (Fig. 4). Thus the proteins that are degraded most rapidly in vitro appear to be most sensitive to proteases.

Although small, such differences in protease sensitivity were observed consistently. This experimental approach necessarily yields only relatively small differences, since it can only examine the changes in trypsin sensitivity upon loss of the most labile proteins. This fraction represents at most about 7–9% of all the cell proteins (i.e., the amount of labeled proteins degraded by the cell during the 2-hr incubation). Growth of cells for an additional 2 hr to select for radioactive proteins with even longer half-lives further reduced the trypsin sensitivity. Analogous results were obtained upon treatment of cell extracts with Pronase at different times after exposure to [3H]leucine (unpublished observations).

DISCUSSION

The present experiments provide strong evidence for a general correlation between rates of degradation of proteins in bacteria and their sensitivity to attack by endopeptidases. Various experimental treatments that promote protein catabolism in vivo also were found to increase the sensitivity of average cell proteins to trypsin or other proteases, including (i) incorporation of several amino acid analogs, such as canavanine, S-(β-aminoethyl)-cysteine, α-amino-β-chlorobutyric acid, azatryptophan, fluorotyrosine, and fluorophenylalanine (Fig. 1, Table 1, and unpublished observations); (ii) premature termination of the polypeptide chain, resulting from incorporation of puromycin (Fig. 1); or (iii) frequent errors in synthesis resulting from a mutation affecting ribosomal structure (ram) (Fig. 2).

These studies thus offer a simple mechanism through which conformation may determine the half-life of cell proteins in vivo. Several findings strongly suggest that the increased protease sensitivity observed under these conditions is causally related to the changes in intracellular degradation. When the bacteria degraded the abnormal analog-containing proteins, the cell-free extracts decreased concomitantly in their sensitivity to proteases (Fig. 2). Eventually the degradative rate in the cells that had been exposed to the analogs returned to control levels, presumably as a result of the selective loss of the abnormal components. Extracts prepared at this time no longer appeared more sensitive to trypsin than
extracts from control cells (Fig. 2). Thus those proteins that were degraded most rapidly by the cells appear to have been responsible for the increased protease sensitivity. In related studies, we have found that those aminoacid analogs whose incorporation promoted intracellular proteolysis to the greatest extent also appear to have the greatest effects on sensitivity to trypsin.

In addition to these observations with abnormal proteins, evidence has been presented that normal proteins that turn over rapidly in growing cells are on the average more sensitive to trypsin or Pronase than more stable cell constituents (Fig. 4). As was observed with the abnormal proteins, the loss of the most labile proteins through degradation in vivo coincided with the loss of those proteins most easily digested by trypsin and Pronase. These findings strongly suggest that the variations in half-lives of normal proteins in E. coli are also determined by their different tertiary conformations, which presumably determine protease sensitivity. In fact, in recent experiments, I have found that treatment of extracts with mild denaturing agents can reduce the observed differences in protease sensitivity between the labile and stable cell constituents.

One possible trivial explanation for the observed correlation between degradative rates in intact cells and protease sensitivity of extracts could be that extracts from cells more active in catabolizing proteins would contain greater amounts of partially digested proteins. Such “slightly chewed” molecules are likely to be more sensitive to further attack by endoproteases. If they accumulated to an appreciable extent in vivo, they might influence the overall protease sensitivity of the extracts. However, analysis of cell extracts with acrylamide gel electrophoresis have not indicated any measurable differences in average protein size after incorporation of a normal amino acid or an analog. Furthermore, all experiments reported here were done with such high protease concentration that the rate of protein hydrolysis in the extracts was far more rapid that that which occurred in vivo [e.g., in normal cells 5-7% of the labeled proteins were degraded per hr, while in our experiments with trypsin most of the radioactive proteins in the extract were hydrolyzed within 45 min (Fig. 4)]. As a result, any partially digested polypeptides present in the extracts could not constitute a significant fraction of the proteins being hydrolyzed.

Evidence for a general correlation between intracellular half-lives and protease sensitivity has been previously lacking, although this possibility has been suggested (1, 4, 10). In related studies, I have also found that a mutant lac repressor (L-1), which is rapidly degraded by the cell, is far more easily digested by several proteases than the wild-type repressor, which is stable in vivo. Marked differences in protease sensitivity were found, even though these proteins do not differ in their ability to bind inducer or in their temperature sensitivity. In related experiments analogous to Fig. 4, I have demonstrated a correlation between protease sensitivity and average rates of protein catabolism in rat liver and rat kidney. In both tissues, those proteins turning over most rapidly appear most sensitive to trypsin and Pronase. These latter findings are in general accord with recent experiments of Bond (10), who demonstrated that the relative sensitivity of five enzymes in rat liver to inactivation by trypsin or chymotrypsin correlated with their relative rates of degradation. However, in her experiments, sensitivity of these five enzymes to Pronase did not correlate simply with the in vivo data.

Substrates or cofactors can protect enzymes from proteolytic attack, and this effect has frequently been suggested as a possible mechanism through which such factors might influence intracellular enzyme levels (10-12). This possibility
appears especially likely in light of my findings that the average stability of cell proteins correlates with their protease sensitivity. However, it is unlikely that this simple correlation by itself can explain all the specificity in the regulation of protein degradation. For example, in certain physiological states (13) cells may selectively degrade proteins that are quite stable under other conditions, possibly through highly specialized proteolytic systems (14). In addition, a specific polypeptide can have very different half-lives in different body tissues (15), possibly because of differences in tissue proteases.

Denatured proteins are more sensitive to proteolytic attack than native enzymes (16). This difference may provide cells with a simple mechanism for the selective degradation of abnormal proteins, such as those arising by mutation, mistakes in translation, intracellular denaturation, etc. Presumably, normal cell proteins share certain conformational features that protect them from rapid hydrolysis under most conditions. In the present studies, differences in protease sensitivity were observed with several endopeptidases of different specificities (e.g., trypsin and chymotrypsin) or even low specificity (e.g., subtilisin and Pronase). Because of these similar findings with such different enzymes, it is likely that rather marked differences in conformation or thermodynamic stability exist between normal and abnormal proteins. Presumably the structural differences that distinguish short-lived normal enzymes from more stable ones are similar in character, although perhaps more subtle. The identification of the structural features responsible for such differences in protease sensitivity may prove of appreciable import for understanding the regulation of enzyme levels.

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