Characterization and Regulation of Protease Synthesis and Activity in *Bacillus licheniformis*

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Received for publication 8 September 1970

Extracts of growing and sporulating cells contain a protease activity that has a broad pH optimum and an unusually broad specificity. The activity, which resides in at least two protein fractions, hydrolyzes all peptide bonds and can reduce a mixture of proteins into a mixture of free amino acids with a high efficiency. No inhibitors of the activity were found, but the protease showed a definite preference for denatured protein as substrate. The synthesis of the intracellular protease activity is under catabolite repression control, as is the extracellular activity. However, the synthesis of the two activities is not coordinate, making the relationship between the two unclear. Due to (i) the specificity of the intracellular activity, (ii) the fact that it is synthesized most rapidly under slow or nongrowing conditions, and (iii) our inability to measure in vivo protein turnover in cells containing high levels of enzyme, a scavenger role is postulated for the enzyme. The rate of protein turnover is not a function of the protease content of the cells.

The relationship between protease biosynthesis and activity and the process of sporulation in *Bacillus* species has not been established unequivocally. In reviewing physiological and genetic data from many laboratories, Schaeffer (18) concluded that extracellular protease activities are not essential for sporulation, and suggested that one or more intracellular proteases must be responsible for protein turnover. However, Mandelstam and Waites (12) stated that "the exoprotease is responsible for the intracellular turnover of protein"; but neither offered a reason for protein turnover in the process of sporulation.

Although the extracellular activity has no apparent function in sporulation, the enzyme molecules may have some relationship to their intracellular counterparts. The latter may be required to (i) degrade selected enzymes to allow for the regulation of metabolism (5); (ii) provide oxidizable monomers for cell energetics (3); (iii) provide monomers for protein synthesis; or (iv) degrade sporulation inhibitors (12). It has been implied (12) that changes in the amounts of activity of extracellular proteases are directly related to changes in intracellular turnover rates, as mutants have been isolated that lose both coincidentally and revertants regain both. However, other mutants exist that lack extracellular protease activity but are still able to turn over protein (18). Thus the absolute relationship between the two proteases does not seem to be direct, although correlations in control may exist (6).

Catabolite repression plays a major regulatory role during sporulation (5, 18). The synthesis of the extracellular protease of *B. licheniformis* is under catabolite repression control (2, 10). With the exception of one preliminary report in which it is suggested that the regulation of the synthesis of the intracellular activity is different from the regulation of the synthesis of the extracellular activity (6), little is known about the control of the synthesis or activity of intracellular proteases. In addition, a number of workers have shown that extracellular protease activity may reside in more than one enzyme species in several *Bacilli* (8, 13, 14), but this information is not available for the intracellular activity.

We report here experiments on the regulation of the synthesis and activity of the intracellular protease, the profile of reaction products, and preliminary work on the separation of the activity into chromatographically distinct fractions. In addition, a role is postulated for the intracellular protease activity.

**MATERIALS AND METHODS**

**Growth conditions.** *B. licheniformis* strain A-5 was used throughout this investigation. Cells were grown on either of two salts media, B salts or T salts. The B salts medium was described by Bernlohr (4). The T salts medium consisted of (per liter): 150 mg of MgSO₄·7H₂O, 125 mg of MgCl₂·6H₂O, 1 mg of MnCl₂·4H₂O, 50 mg of CaCl₂·2H₂O in 65 mm potassium phosphate buffer, pH 6.9. One-liter cultures, grown for protease extracts, consisted of the T salts...
medium supplemented with 20 mM glucose plus 10 mM NH₄Cl, or with 50 mM glutamate, or on a medium consisting of 0.5% peptone, 0.15% yeast extract, and 0.25% NaCl with or without 20 mM glucose. For the determination of turnover, 10-ml cultures were grown with the B salts medium supplemented with either 20 mM glucose plus 50 mM NH₄ lactate or with 50 mM glutamate. All cultures were grown at 37°C.

Preparation of extracts. Extraction of the intracellular enzyme from cells, and all subsequent steps, were carried out at 0 to 5°C. Cells were harvested by centrifugation at 13,000 × g for 20 min and washed with water. Packed cells from a 1-liter culture were suspended in 5 ml of water, and broken by sonic treatment for 1 min in a 20.0-kc MSE (Measuring & Scientific Equipment) sonic oscillator. The broken cell suspension was centrifuged for 45 min at 40,000 × g in a Sorvall RC-2B centrifuge. The supernatant solution was then dialyzed against two changes of 2 liters of water for 2 hr.

To prepare the extracellular enzyme, the 13,000 × g supernatant solution from 1 liter of culture was concentrated to 10 ml on a Rinco rotary evaporator and then dialyzed as above.

Diethylaminoethyl cellulose chromatography. A column (1.5 by 19 cm) was prepared with washed Whatman DE-52 cellulose and equilibrated with 0.005 M tris(hydroxymethyl)amino methane hydrochloride buffer, pH 8.0. The void volume of the column was 15 ml, and 5-ml fractions were collected. Crude intracellular extract (18.0 mg of protein) was absorbed, and the column was washed with 60 ml of the equilibrating buffer. A linear gradient from 0 to 0.025 M KCl was initiated. After 100 ml of the gradient volume had passed through the column, a stepwise elution was initiated, consisting of 20-ml volumes of buffer containing 0.1 M KCl, 0.2 M KCl, and 0.5 M KCl, in that order.

Enzyme assays. Both the tube assay and the dialysis assay consisted of measuring the release, in acid-soluble form, of radioactive material from 14C-protein at 37°C.

Preparation of 14C-protein. B. licheniformis cells were grown in a 1-liter culture containing 20.0 ml glucose, 10.0 mM NH₄Cl, and T salts, with the addition of 150 μCi of 14C amino acids. When the culture optical density reached 2.5 (0.5 mg of dry weight/ml), 10 mg of lysozyme was added to the culture. After 45 min of incubation at 37°C, 100% trichloroacetic acid was added to a final concentration of 5%. After storage overnight at 4°C, the precipitated protein was centrifuged and washed three times with 25 ml of 5% trichloroacetic acid at 100°C, twice with 25 ml of CHCl₃-

CH₂OH (1:1) at 50°C, and once with 25 ml of CH₂OH at 50°C. After the protein had been dried by vacuum desiccation, it was taken up in 0.1 ml Tris-hydrochloride buffer, pH 9.0. The pH was adjusted to 9.0 with Na₂CO₃, stirred at 60°C for 1 hr, and further solubilized by sonic treatment for 5 min. After centrifugation at 40,000 × g for 20 min, the protein concentration was determined by the method of Lowry et al. (11). This protein had a specific radioactivity in the range of 200 counts per min per μg, by using the counting system previously described (4).

Tube assay. The reaction mixture, containing 4 mg of 14C-protein, 1 mg of protease extract, and 0.1 ml Tris-hydrochloride buffer, pH 9.0, in a final volume of 1.0 ml, was incubated at 37°C. Samples of 0.1 ml were removed at 10-min intervals from 0 to 60 min, and added to 0.9 ml of 5% perchloric acid. After centrifugation, 0.1 ml was removed and placed in a plastic scintillation vial, and 10 ml of scintillation fluid (4) was added. The radioactivity of the sample was determined in a Beckman LS-100 scintillation counter.

Fractions from the diethylaminoethyl column were assayed by combining 0.1 ml of eluate with 0.16 mg of 14C-protein. After incubation for 20 min at 37°C, 5% perchloric acid was added to a final volume of 1.0 ml, and the above procedure was followed.

Dialysis assay. A standard dialysis bag containing 4 mg of 14C-protein and 1 mg of protease extract, adjusted to a final volume of 1.0 ml, was placed in a beaker containing 50 ml of 0.1 M Tris-hydrochloride buffer, pH 9.0, and incubated with shaking at 37°C. At various times, 0.1 ml of the dialysate was removed and the radioactivity was determined as above.

Amino acid assay. Ninhydrin reacting material was measured by a modification (4) of the method of Trol and Cannan, and quantitative amino acid analysis was by use of a Beckman model 120 B amino acid analyzer as previously described (4).

Uptake and turnover. Uptake and turnover studies were performed as previously reported by Bernlohr (4).

Materials. The 14C-t-amino acid mixture was purchased from Amersham/Searle Corp.; it consisted of 14 t-amino acids at a specific activity of 52 mCi/mmol of carbon. This mixture was used in the preparation of 14C-protein and in the turnover experiments. All other chemicals were of reagent grade purity.

RESULTS

Characterization of the protease activities. Although it is clear that the extracellular protease activity is not directly involved in the sporulation process, a great fund of information exists on the multiplicity, activity, and physical characteristics of these enzymes from a number of Bacillus species (18). Because of this, many of the experiments were performed on both the intracellular and the extracellular (E) activities.

Most assays for protease activity involve a measure of the rate of solubilization of a precipitable protein. We investigated the time course of release of perchloric acid-soluble material from radioactive acid-washed B. licheniformis protein, and the appearance of dialyzable peptides and amino acids. Figure 1 presents the results of typical experiments. The "tube assay" of the I and E protease activities (Fig. 1A), which measures acid-soluble material, produces data that are significantly different from those of the dialysis assay. The E activity solubilizes protein rapidly, but does not produce free amino acids to any extent. The I activity solubilizes protein at a lower rate, but significant quantities of free amino acids are released. Comparative specific activities for protein solubilization are about 300 μg of protein
solubilized per min per mg of E protein versus 10 
µg per min per mg of I protein, a 30-fold difference. However, 1 mg of I protein is found to re-
lease at least three times as much free amino acid as a percentage of the solubilized material, as
does 1 mg of E protein. Thus, neither criterion alone is satisfactory for describing the rate of the reaction by either protease preparation.

The dialysis assay measures the release of dialyzable material from standard dialysis bags ins-
ide of which are placed the enzyme and sub-
strate. Figure 1B shows that, after a 20- to 30-
min lag, the E activity causes the release of dialyzable material at a rate that is two to three
times faster than the I activity on a specific ac-
tivity basis. The profile of events inside the dial-
ysis bag is shown in Fig. 2 for the I activity. Acid-insoluble protein (acid-washed casein) de-
creased to about 10% of the original level in 2 hr.
Total protein decreased at about one-half this rate, and the difference between the two is plotted as acid-soluble protein (peptide) that reaches a plateau level in about 2 hr. The acid-soluble but nondialyzable material decreased slowly as free amino acids accumulated. Added to this figure is the time course of release of ninhydrin-reacting material outside the bag, showing that little free amino acid is detected until most of the protein inside the bag is solubilized. The protease en-
zymes are sensitive to autodigestion and, in the case of the experiment shown in Fig. 2, subse-
quent addition of more substrate at 4 hr does not lead to further release of peptides or amino acids.

The I activity releases a complete mixture of amino acids from denatured B. licheniformis pro-
tein or from acid-washed casein. Table 1 shows the amino acid analysis data from experiments in
which the material outside of dialysis bags was concentrated and analyzed. When compared with the known amino acid composition of the enzyme preparation, (column 1), the autodigested prepa-
ration (column 2) and the denatured protein plus I activity experiments (column 3) show that all amino acids are released and that the yields of

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Fig. 1. Comparison of the activities of the I protease and E protease in the two assay systems. The E protease (●) assay contained 4 mg of "C-protein and 0.4 mg of extract; the I protease (○) assay contained 4 mg of "C-protein and 1.0 mg of extract. The total free amino acid released after 60 min, (▲) E protease and (Δ) I protease, is weight/weight per cent.

Fig. 2. Dialysis assay of intracellular protease ac-
tivity. Total protein (●), acid-insoluble protein (○), acid-soluble protein (△), ninhydrin-reacting material (▲). Reaction mixture contained 4 mg of casein and 1 mg of I protease extract.
each are fairly good. The hydrolysis of prolyl and aspartyl peptides appears to be slow. However, the I activity could function in protein turnover, since peptides are hydrolyzed and would not be expected to accumulate.

The apparent broad spectrum of activity of I preparations is unusual, and this prompted a preliminary characterization, by diethylaminoethyl cellulose chromatography, of the activity isolated from post-log-phase cells. Figure 3 shows the results of one such experiment. The protease activity migrated as two components, a weakly bound (fraction B) and a tightly bound (fraction C) form. Fraction A eluted with the void volume and cochromatographs with fraction B when smaller amounts of protein are applied to the column. Both enzyme fractions catalyze the release of free amino acids from denatured \textit{B. licheniformis} protein; as yet no difference between the two is apparent. In all chromatographic experiments the sum of fractions A and B contained about 50\% of the activity of fraction C. It is tentatively concluded that the I activity of \textit{B. licheniformis} is the result of two or more proteolytic enzymes, one of which predominates.

**Regulation of the I and E enzymes.** Cells were grown on four media that produced significantly different growth rates. Generation times, as shown in Table 2, varied from 180 min for glutamate-grown cells to 40 min for cells grown on peptone, yeast extract, and glucose. Catabolite repression of the arginine degradative pathway is not operative in slowly growing cells (glutamate) but is strongly effective in cells growing at generation times of 60 min or less (10). Table 2 shows that the activities of the I and E enzymes vary over a wide range (up to 100-fold) and that both activities are under catabolite repression control. The activities were measured in preparations taken at three different times during the growth cycle of the cells. These times are shown in Fig. 4 and are designated as mid-log phase, late-log phase, and sporulating or post-log phase. It is clear that fast-growing mid-log-phase cells contain the lowest I activities, whereas slow-growing cells contain the highest. Because the tube assay for protein solubilization was utilized for these experiments, the E activity is apparently higher, but this has little meaning as discussed earlier. The amount of E activity is directly related to catabolite repression, but the profile of activities shown in Table 2 does not change exactly in parallel to the I activities. For example, the E activities from sporulating cells decrease in left to right reference in Table 2, whereas the I activities do not.

The regulation of the activity of the I and E enzymes was examined. The pH optimum of each was the same, being very broad and constant between pH 7.0 and 9.5. A pH of 9.0 was chosen for all assays because this pH permitted a greater solubility of denatured protein.

A series of possible physiological inhibitors of both the I and E activities was tested at concentrations that were considered to be as high as those that could exist in vivo. Table 3 shows that none had an effect on the I activity that is great enough to afford a significant intracellular regulation. Thus, the I activity may not be regulated by an inhibitory or stimulatory mode. However, it was thought possible that the I activity may have some specificity in regard to substrate. A radioactive protein extract was prepared by using as much care as possible to preclude denaturation, and this "native protein" was compared with denatured protein as a substrate. Figure 5 shows that this "native protein" was hydrolyzed at less then one-third the rate under otherwise identical conditions. In addition, the I activity did not inactivate measurable amounts of partially purified pyruvate kinase or phosphofructokinase in 30 min under conditions in which denatured protein is degraded at a rate of 7 mg of protein per min per mg of I protein (unpublished data, F. W. Tuominen and C. K. Marschke). The I enzyme used was from fraction B of Fig. 3. Thus, it appears that the I activity has a definite preference for denatured protein and would be expected to degrade active enzymes very slowly in vivo.

**Role of the I activity in turnover.** Extracts of mid-log-phase cells grown on glutamate exhibit a

### Table 1. Amino acid composition of the protease extract, and analysis of the solubilized products of the protease activity

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Acid-hydrolyzed protease (18 hr)</th>
<th>Autodigested protease (18 hr)</th>
<th>Protease-hydrolyzed \textit{B. licheniformis} protein (2 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine . . .</td>
<td>8.2*</td>
<td>12.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Histidine  . . .</td>
<td>2.1</td>
<td>3.4</td>
<td>1.9</td>
</tr>
<tr>
<td>Arginine . . .</td>
<td>4.5</td>
<td>7.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Aspartate  . . .</td>
<td>9.5</td>
<td>2.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Threonine . . .</td>
<td>4.9</td>
<td>4.4</td>
<td>5.1</td>
</tr>
<tr>
<td>Serine . . .</td>
<td>4.6</td>
<td>8.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Glutamate . . .</td>
<td>11.3</td>
<td>11.9</td>
<td>17.9</td>
</tr>
<tr>
<td>Proline . . .</td>
<td>4.3</td>
<td>0.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Glycine . . .</td>
<td>9.5</td>
<td>5.1</td>
<td>6.9</td>
</tr>
<tr>
<td>Alanine . . .</td>
<td>10.6</td>
<td>9.9</td>
<td>14.0</td>
</tr>
<tr>
<td>Valine . . .</td>
<td>6.9</td>
<td>7.6</td>
<td>8.0</td>
</tr>
<tr>
<td>Methionine . . .</td>
<td>2.2</td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Isoleucine . . .</td>
<td>5.8</td>
<td>5.3</td>
<td>5.2</td>
</tr>
<tr>
<td>Leucine . . .</td>
<td>8.7</td>
<td>10.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Tyrosine . . .</td>
<td>2.9</td>
<td>4.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Phenylalanine . . .</td>
<td>4.0</td>
<td>4.4</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* All results indicate moles per cent.
protease activity that is about 15 times that of extracts from mid-log-phase cells grown on glucose. Since protein turnover is thought to be in the range of 1 to 3% per hr in growing bacterial cells (15), the possibility existed that greater turnover might be demonstrable in cells containing high protease activity, but not in cells with low activity. The activity in cells grown on glutamate (Table 2; 48 µg per min per mg) has an activity that could cause a turnover rate of about 288% per hr (if the protein inside the cell is attacked at a rate similar to that shown for "native protein," the rate would be about 100% per hr; Fig. 5).

Separate mixtures of amino acids were added to cells growing on glucose (Fig. 6A) and glutamate (Fig. 6B). The majority of this amino acid was incorporated into protein, and a large excess...
of an unlabeled amino acid mixture was then added. No loss of radioactive amino acid was noted from either culture, showing that no measurable turnover could be demonstrated. A parallel set of experiments was performed by using cells incubated in the presence of chloramphenicol (Fig. 6C and 6D) in which significant pools of amino acids accumulated and from which radioactive amino acid was chased. Thus, if turnover was occurring in either culture at a rate consistent with the resident protease activity, it could have been measured. It is clear that the I activity is not functioning in vivo.

**DISCUSSION**

The intracellular protease activity of *B. licheniformis* has an unusually broad specificity, producing a high yield of all amino acids. This behavior is similar to that of Pronase, an extracellular enzyme from *Streptomyces griseus* (19), and can be matched only by a mixture of other hydrolases (1). The I activity and Pronase both hydrolyze all peptide bonds, and in both cases release proline and aspartic acid most slowly (Table 1). This broad specificity leads to ambiguity in regard to determining quantitative values for the rate of the reaction catalyzed by the enzymes. In the case of the I activity, varying rates are observed when different proteins are used as substrate, and it is assumed that proteins with high proline (or hydroxyproline) and aspartic acid contents would be hydrolyzed slowly.

It has been reported that extracellular proteases are under catabolite repression control (10, 18), but reports of amino acid repression control have appeared (6, 18). This latter phenomenon may be catabolite repression, in reality, as amino acid stimulation has been observed in media devoid of glucose (7). Table 2 shows that the biosynthesis of the I activity is under catabolite repression control. Although the E activity has been shown to be under similar control (10), an examination of Table 2 reveals that the regulation of the synthesis of the two is not coordinate. Thus, sporulating cells grown on peptone-yeast extract contain four to five times the level of I activity as sporulating glucose-grown cells, whereas the E activity from the same cultures

![Figure 4](http://jb.asm.org/)

**FIG. 4.** Typical growth curve for *B. licheniformis*, showing times when samples were taken for data in Table 2. Generation times are shown in Table 2.

**Table 3.** Effect of various compounds on protease activities

<table>
<thead>
<tr>
<th>Addition*</th>
<th>Concentration</th>
<th>V/Vc°</th>
<th>V/Vc°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(intra-cellular)</td>
<td></td>
<td>(extra-cellular)</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Amino acid mixture</td>
<td>5 mg/ml</td>
<td>1.35</td>
<td>1.03 (2.5 mg/ml)</td>
</tr>
<tr>
<td>Glucose</td>
<td>5 mg/ml</td>
<td>1.01</td>
<td>0.70</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 mg/ml</td>
<td>1.00</td>
<td>1.15</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>5 mg/ml</td>
<td>1.51</td>
<td>0.68</td>
</tr>
<tr>
<td>Tricarboxylic acid cycle intermediate</td>
<td>4 mg/ml</td>
<td>1.00</td>
<td>0.49</td>
</tr>
<tr>
<td>ATP</td>
<td>10 mM</td>
<td>0.94</td>
<td>0.90</td>
</tr>
<tr>
<td>ADP</td>
<td>10 mM</td>
<td>0.94</td>
<td>0.86</td>
</tr>
<tr>
<td>AMP</td>
<td>10 mM</td>
<td>1.00</td>
<td>0.88</td>
</tr>
<tr>
<td>Cyclic-AMP</td>
<td>0.5 mg/ml</td>
<td>1.14</td>
<td>0.99</td>
</tr>
<tr>
<td>Acid-soluble protein</td>
<td>0.8 mg/ml</td>
<td>1.07</td>
<td>0.75</td>
</tr>
<tr>
<td>Aacetate</td>
<td>5 mg/ml</td>
<td>0.73</td>
<td>2.00</td>
</tr>
</tbody>
</table>

*a Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

° Vc, reaction rate in absence of effectors; V, reaction rate in presence of effectors.

The mixture included 0.8 mg each of malate, fumarate, succinate, citrate, and α-ketoglutarate per ml.

D Soluble material accumulating inside dialysis bag; see Fig. 2.

![Figure 5](http://jb.asm.org/)

**FIG. 5.** Comparison of activity of I protease with denatured protein or native protein as substrate. One milligram of extract protein used in both assays. The substrate concentration was 4.0 mg denatured (●) or 1.0 mg native (○) protein. The substrate is saturating in both cases.
Fig. 6. Efflux systems of B. licheniformis and the absence of measurable turnover. ¹⁴C-amino acid (1.25 µCi) was added at zero time. C and D, Treated with 100 µg of chloramphenicol (CHLORAM) per ml at 2 min before zero time. At 8 min, 100 µg of Casamino Acids per ml was added to each culture. A and C, Log-phase cultures grown on glucose NH₄Lac-B salts; B and D, log-phase cultures grown on glutamate and B salts. Symbols: ○, buffer washed; △, buffer washed after addition of Casamino Acids; ○, hot trichloroacetic acid-washed; △, hot trichloroacetic acid-washed after addition of Casamino acids.

exhibit a three-fold difference in the other direction. This noncoordinate phenomenon can be explained in at least two ways. First, these data could be interpreted to show that there is no relationship, either in control or in terms of primary structure, between the I and E enzymes. This seems unlikely, because several laboratories have reported that single-step mutants can lose a primary extracellular protease and the ability for intracellular protein turnover and sporulation concurrently (12, 18), although all do not.

A more plausible suggestion would involve the regulation of the transport of all or part of one or more intracellular enzymes into the external medium. This type of phenomenon, a catalyzed release of enzyme, has been observed for extracellular penicillinase synthesis in B. licheniformis (17). Here, the intracellular form has a greater
molecular weight than the extracellular form, and it is clear that there is an intermediate bound form, the release of which may be separately regulated (18). We have found (unpublished data) that the I protease activity will elute with the void volume from a Sephadex G-100 column, whereas the E activity is bound to Sephadex G-25, suggesting that the activities reside in molecules of significantly different molecular weight. Thus, the cell may be able to regulate the release of enzyme from the cell.

Cells growing in mid-log phase on glucose contain an I activity of about 4 μg per min per mg (Table 2), which is equivalent to 20 to 25% hydrolysis of all resident protein per hour. If the protease was active in vivo, an appreciable turnover of protein might be expected, but in many cases has not been observed (see references in 15).

In fact, we have come to believe that the protein turnover rate in growing cells does not exceed 3% per hr. Using rather crude methods, we were able to show that the turnover rate in growing B. licheniformis cells was less than 10% per hr (Fig. 6) even in cells containing a protease activity equivalent to 288% per hr (Table 2). Thus, the intracellular activity is not fully active in vivo.

A search was made for possible inhibitors of the I activity based on the hypothesis that the cell regulates in vivo turnover by some form of feedback inhibition. No strong inhibitors were found (Table 3), even though a wide range of candidates was used. This result led to a study of the role of the substrate in determining the apparent reaction velocity. It was shown that the I activity hydrolyzed "native" protein at a much lower rate than "denatured" protein (Fig. 5) and affected the activity of purified pyruvate kinase less than 1% over a 30 min-period (unpublished data, F. W. Tuominen). This result was not unexpected, as it has been shown that several proteases hydrolyze proteins with "ordered structure" (high α-helix and cross β-structure content) poorly (9). It is concluded that the intracellular protease activity of B. licheniformis "strongly prefers" denatured proteins as substrate.

This conclusion places the role of the I activity more into the realm of a scavenger than of a turnover enzyme. The results also show that the rate of turnover is apparently regulated by a mechanism other than the simple biosynthesis of a hydrolytic enzyme. Since protein turnover increases significantly to a value of approximately 20% per hr during sporulation in B. licheniformis (manuscript in preparation) and 18% per hr in B. subtilis (20), this rate may be the result of an increased rate of protein denaturation under non-growing conditions (21).

ACKNOWLEDGMENTS
This research was supported by Public Health Service grants AI-05096 from the National Institute of Allergy and Infectious Diseases, Research Career Development Award GM-KJ-7709 from the National Institute of General Medical Sciences, and by grant GB-7347 from the National Science Foundation.

The assistance of B. H. Gray, N. Minahan, and L. Larson is greatly appreciated.

LITERATURE CITED