JOURNAL OF BACTERIOLOGY, Aug. 1983, p. 522–530
0021-9193/83/080522-09$02.00/0

Vol. 155, No. 2

Degradation of Ornithine Transcarbamylase in Sporulating *Bacillus subtilis* Cells

JUSTIN O. NEWAY† AND ROBERT L. SWITZER*

Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

Received 13 January 1983/Accepted 2 May 1983

When *Bacillus subtilis* cells grew and sporulated on glucose-nutrient broth, ornithine transcarbamylase (OTCase) was synthesized in the early stationary phase and then inactivated. The loss of OTCase activity was much slower in a mutant that was deficient in a major intracellular serine protease (ISP). Immunochemical analysis showed that synthesis of OTCase decreased to a low, but detectable, level during its inactivation and that loss of activity was paralleled by loss of cross-reactive protein. Because the antibodies were capable of detecting denatured and fragmented forms of OTCase, we conclude that inactivation involved or was rapidly followed by degradation in vivo. Native OTCase was not degraded in crude extracts or when purified ISP and OTCase were incubated together under a variety of conditions. Synthesis of OTCase was not shut off normally in the ISP-deficient mutant. When the effects of continued synthesis were minimized, OTCase was degraded only slightly slower in the mutant than in its parent. Thus, the mutant had unanticipated pleiotropic characteristics, and it was unlikely that ISP played a major role in the degradation of OTCase in vivo.

The regulation of metabolism by enzyme inactivation is a well-known phenomenon in microbes (20), but in only a few cases has it been shown that the inactivated enzymes are degraded. Previous studies from this laboratory have identified two examples of degradative inactivation in *Bacillus subtilis*. Aspartate transcarbamylase, the first enzyme of pyrimidine biosynthesis de novo, is rapidly inactivated at the end of exponential growth (4, 22). This is also the case for glutamine phosphoribosylpyrophosphate amidotransferase (21). In both of these cases studies of the fate of the enzyme protein, using monospecific antibodies, have shown that inactivation is accompanied by the loss of cross-reacting material (CRM) (9, 15). Characterization of the specificity of the antibodies used showed that they were capable of detecting denatured, chemically modified, and proteolytically degraded forms of their respective antigens. This suggests that inactivation of these enzymes in vivo involves or is rapidly followed by extensive proteolysis. In neither of these two cases has it been possible to identify the protease or proteases responsible for the degradation of the enzymes in vivo.

Deutscher and Kornberg (4) showed that ornithine transcarbamylase, (OTCase; EC 2.1.3.3) activity was lost from crude extracts of *B. subtilis* cells several hours after the end of exponential growth in nutrient broth medium. They also showed that the enzyme was not present inside mature spores. Setlow and Primus (16) showed that OTCase activity could not be found in spores of *B. megaterium* and that a period of protein synthesis was required before the activity appeared again after germination. Paulus and Switzer (13) reported that OTCase activity was not excreted into the medium during inactivation of the enzyme in vivo. These authors also observed that the loss of OTCase activity from sporulating cells of *B. subtilis* was fourfold slower in a protease-deficient mutant, strain S-87 (5), than it was in the parent strain 168 (13). The rates of inactivation of aspartate transcarbamylase, glutamine phosphoribosylpyrophosphate amidotransferase, and both the arginine- and uracil-repressible carbamyl phosphate synthetases were similar in this mutant and its parent. Hageman and Carlton (5) have shown that strain S-87 lacks an intracellular serine protease (ISP). Although it has not been shown that strain S-87 is mutant in the structural gene for ISP, the results suggested that ISP might be directly responsible for the selective inactivation of OTCase in *B. subtilis*. The objectives of the work presented in this study were to characterize further the inactivation of OTCase in vivo, and, using purified OTCase and ISP, to attempt reconstruction of the inactivation in vitro.

† Present address: Genetics Institute, 225 Longwood Ave., Boston, MA 02115.

MATERIALS AND METHODS

Microbiological methods. *B. subtilis* strain S-87 (Trp^-, protease deficient) and its parent strain 168 (Trp^-) were obtained from J. H. Hageman (5). Unless otherwise stated, all bacteria were grown in supplemented nutrient broth (4) containing 0.1% (wt/vol) glucose at 37°C with vigorous shaking (300 rpm; 500 ml of culture in a 2-liter flask).

Assays. Procedures for harvesting, washing, and extracting cells, protein assays, and OTCase assays are described in the accompanying paper (11). ISP was assayed with Azocoll (Calbiochem) as the substrate. The reaction mixture contained 1.5 ml of 0.5 M Tris-hydrochloride buffer, pH 7.5 (at 37°C), 1 ml of a 15-mg/ml portion of Azocoll, 2 mM $CaCl_2$, and cell extract to 3 ml. The reaction was initiated by the addition of cell extract and was incubated in a vigorously shaking 25-ml test tube in a 37°C water bath. After 40 min, reactions were quenched by the addition of 1 ml of 30% (wt/vol) trichloroacetic acid. The optical density at 520 nm was measured after 0.4 ml of 5 M NaOH had been added, and the remaining Azocoll was removed by centrifugation in a clinical centrifuge. The color released from total digestion of Azocoll was measured by allowing an assay described above to go to completion (12 to 16 h). The scatter between duplicate values could be considerably reduced by slow freeze-thaw of the substrate solution (−20°C to room temperature) before use. One unit of ISP activity was defined as that amount of enzyme that released 1 μg of Azocoll to the acid supernatant solution per min at 37°C.

Purification of ISP. ISP was purified from *B. subtilis* 168 that was grown so as to obtain maximal production of activity. The bacteria were inoculated from a 24-h nutrient agar plate into ten, 25-ml test tubes each containing 10 ml of glucose-nutrient broth. These starter cultures were grown at 37°C with shaking at 300 rpm for 8 h and were then each used to inoculate 1 liter of nutrient broth containing 0.1% glucose in a 2-liter Erlenmeyer flask. Flask cultures were grown at 37°C for 18 to 20 h with shaking at 300 rpm. Cells were harvested and washed by centrifugation to remove extracellular proteases as described previously (11). This procedure gave 45 g of washed cells with a high specific activity of ISP. Cells were stored as a frozen paste at −20°C until used. ISP was purified by a combination of the methods reported by Strongin et al. (19) (see also references 12, 17, 18; J. O. Neway, Ph.D. thesis, University of Illinois, Urbana, 1983). The purified ISP was 400-fold enriched over the crude extract and had a specific activity of 1,300 U/mg. It was essentially free of contaminants and resolved into two protein bands of M_r = 35,000 and 36,000 ± 3,000 on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Sucrose density gradient centrifugation yielded an M_r of 70,000 for the native enzyme. These results were consistent with the findings of Strongin et al. (19), who showed that the smaller protein band was a cleavage product derived from the larger protein band.

Other methods. Procedures for purification of *B. subtilis* OTCase, preparation of anti-OTCase antibodies, and measurement of OTCase CRM by labeling of cultures with [^{3}H]leucine, immunoprecipitation, and analysis of the immunoprecipitates by SDS-polyacrylamide gel electrophoresis are described in the accompanying paper (11).

To measure the rate of OTCase synthesis in vivo, 500-ml cultures were grown as described above. At various times during growth, a 32- or 21-ml sample was removed and placed in a 125-ml Bellco flask, and 0.3 mCi of L-[4,5-^{3}H]leucine (60 Ci/mmol) was added with a sterile syringe. The small flask was then incubated for 20 min at the same temperature and shaking rate as the large parent flask. Two 1- or 0.5-ml samples were removed for determination of total leucine incorporation into protein (see below), and the remainder of the flask contents was harvested by centrifugation and washing three times in the usual manner. Preparation of cell extracts, immunoprecipitation, electrophoretic separation, and counting of gel slices was done as described previously (11). The two 1- or 0.5-ml samples of culture removed from the small pulse-labeled flask described above were each added to 0.33 volume of 40% (wt/vol) trichloroacetic acid in a 1.5-ml Microfuge tube. A 50-μl amount of a 1-mg/ml solution of bovine serum albumin was added, and the mixture was incubated overnight at 4°C. The acid-precipitated material was collected by centrifuging in a Beckman model B Microfuge at top speed for 1 min. The precipitates were washed three times by resuspending in 0.3 ml of 20% trichloroacetic acid, and washed pellets were dissolved by boiling in 0.2 ml of 1 M NaOH for 5 min, acidified by addition of 0.214 ml of 1 M HCl, and counted in 10 ml of scintillation cocktail (1) after 1 ml of water was added to remove turbidity. A 20-μl sample of the acid supernatant resulting from overnight incubation was also counted to correct for small differences in the total amount of [^{3}H]leucine added to each subculture.

For immunoblotting experiments, OTCase fragments were transferred to a sheet of Genescreen (New England Nuclear) by the "western" blotting method of Burnette (3) with the following modifications. Blotting was done in a Transblot apparatus (Bio-Rad Laboratories) operated at 40 V for 20 h. The gel and Genescreen were positioned in the apparatus with three layers of Whatman 3 MM paper on each outer side. After blotting, the Genescreen was treated as described by Burnette (3), except that 0.05% Nonidet P-40 was replaced with 0.05% Triton X-100 and the first wash containing this detergent was lengthened from 20 min to 1 h. The antibody was rabbit anti-OTCase immunoglobulin G (11). The detecting protein was ^{125}I-labeled staphylococcus protein A (the gift of S. Bower) added to the bovine serum albumin-saline buffer at a concentration of 2.25×10^5 dpm/ml. Kodak XAR-5 X-ray film was exposed to the air-dried Genescreen for 17 h at −70°C before development.

[*methyl*-^{14}C]OTCase was prepared by reductive alkylation of 1.8 mg of purified OTCase per ml with 1.25 mM [^{14}C]HCHO (10 μCi/μmol) and 1.25 mM $NaCNBH_3$ according to the procedure of Jentoft and Dearborn (7). The dialyzed product contained two [^{14}C]methyl groups per subunit and retained 65% of its original specific activity.

RESULTS

Inactivation of OTCase in vivo. When *B. subtilis* cells were grown and allowed to sporulate in

a glucose-nutrient broth medium, OTCase synthesis was repressed until the end of exponential growth. OTCase rose to a maximum during the next 4 h of culturing and then declined with a half-life of about 4 h (Fig. 1). The ISP-deficient strain S-87 (5) showed a similar pattern of development, except that OTCase activity declined much more slowly (Fig. 1). As expected (5), protease levels were much lower in extracts of strain S-87; this strain also failed to develop refractile bodies during the period when strain 168 did so.

Addition of 50 mM sodium monofluoroacetate to a similar culture of strain 168 after the loss of OTCase had begun (12 h; see Fig. 1) had no effect on the rate of subsequent loss of OTCase. The second peak of protease activity (at 17 h; see Fig. 1) did not appear after fluoroacetate addition, which showed that the inhibitor did interfere with energy-yielding metabolism, as expected from earlier results (22, 23). It is possible that the deprivation of energy induced by fluoroacetate was sufficient to block protease synthesis but not sufficiently extreme to block degradation. Addition of 100 μg of chloramphenicol per ml at the same time (12 h) to another culture also prevented the second peak of protease activity from appearing, but, again, OTCase activity disappeared at the same rate as in a control culture. These observations suggest that once OTCase inactivation had begun, it was not blocked by inhibition of energy metabolism or protein synthesis. On the other hand, addition of 100 μg of chloramphenicol per ml to a culture of strain 168 during the rise in OTCase activity (8 h; see Fig. 1) blocked any further increase in OTCase and protease activity and greatly inhibited the loss of OTCase activity. Thus, some required protein apparently must be synthesized before OTCase inactivation. This was the case even though the cells contained about half of their maximal protease activity at the time of chloramphenicol addition.

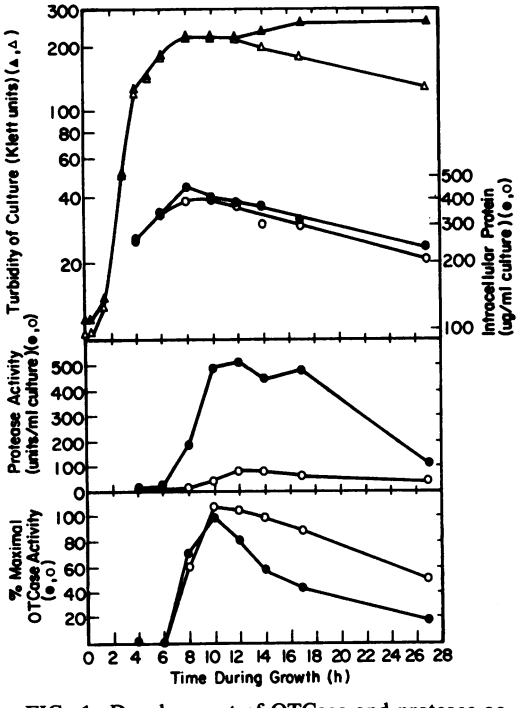

FIG. 1. Development of OTCase and protease activities during growth and stationary phase of *B. subtilis* strains 168 and S-87 on glucose-nutrient broth. Symbols: ●, ▲, strain 168; ○, △, protease-deficient strain S-87; 100% equal 211 mU per ml of culture in both cases.

Characterization of anti-OTCase antibodies. The preparation of monospecific, precipitating antibodies directed against *B. subtilis* OTCase has been described in the accompanying paper (11). For studies of OTCase degradation in vivo, it was necessary to establish the ability of these antibodies to bind and precipitate denatured and fragmented forms of OTCase. Limited digestion of [*methyl*-^{14}C]OTCase with trypsin yielded a cleaved enzyme, which resolved on SDS-polyacrylamide electrophoresis into two fragments with M_r = 30,000 and 14,000 (Neway, Ph.D. thesis). This "nicked" form of the enzyme was precipitated as efficiently as native OTCase by anti-OTCase antibodies. Both fragments were detected on SDS-polyacrylamide gel electrophoresis of immunoprecipitates. However, chromatography of the nicked OTCase on Bio-Gel P-150 showed that the protein still migrated as a native dimer (M_r = 90,000), even when the chromatography buffers contained 4 M urea and 10 mM 2-mercaptoethanol.

Acid-denatured [*methyl*-^{14}C]OTCase was prepared by treating the native radiolabeled protein with HCl at pH 1.5 for 15 min at room temperature and then redissolving the precipitate at pH 10 with NaOH. This material had lost more than 99.5% of its OTCase activity. A quantity of anti-OTCase antibody that precipitated 97% of a 10-μg sample of native [*methyl*-^{14}C]OTCase precipitated about 70% of the acid-denatured material (10 μg) under the usual immunoprecipitation conditions. The efficiency of precipitation of a mixture of 5 μg of native and 5 μg of acid-denatured [*methyl*-^{14}C]OTCase was 93%. Thus, the antibodies were somewhat less efficient in precipitating denatured OTCase, but such material should be detected well in mixtures with native OTCase, which was always included in our protocols for immunoprecipitation from radiolabeled crude extracts.

To test the ability of anti-OTCase antibodies to bind separated and denatured OTCase sub-

units and fragments, OTCase was disgested with trypsin and purified ISP (1% SDS was included when ISP was used). The products were separated by electrophoresis on SDS-containing polyacrylamide slab gels, and their ability to bind anti-OTCase was examined by the western immunoblot technique (3). A control experiment in which [*methyl*-^{14}C]OTCase was digested, electrophoresed, and transferred to the Genescreen before autoradiography established that OTCase and all of its fragments were very efficiently transferred onto the Genescreen. Immunochemical staining with [^{125}I]protein A revealed that the anti-OTCase antibodies used in this study were capable of binding well to the SDS-denatured OTCase subunit and much less efficiently, but detectably, to large denatured OTCase fragments. Small fragments were not detected.

Immunochemical characterization of OTCase inactivation in strain 168. Measurement of the rate of OTCase degradation in the experiments to follow involved addition of L-[4,5-^{3}H]leucine to the culture at an early time. Samples were harvested at various times thereafter, and OTCase CRM was precipitated from the extracts. The immunoprecipitates were analyzed by SDS-polyacrylamide tube gel electrophoresis. To ensure that the rate of loss of CRM so obtained was an accurate measure of the rate of OTCase degradation, it was necessary to demonstrate that synthesis of OTCase was negligible during degradation. If OTCase synthesis continued during degradation, the disappearance of radioactivity from the immunoprecipitates would be artificially slowed by the continued incorporation of [^{3}H]leucine into the enzyme. The rate of OTCase synthesis was measured with anti-OTCase antibody by exposing culture samples to a pulse of [^{3}H]leucine at intervals during OTCase inactivation and measuring the amount of radioactivity incorporated into immunoprecipitated OTCase (Fig. 2). The amount of [^{3}H]leucine incorporated into bulk protein varied during the cultivation. This may have resulted from variations in the rate of uptake of free leucine from the medium, dilution of the isotope with nonradioactive leucine by intracellular synthesis or degradation of peptides from the medium, or changes in the rate of total protein synthesis. To obtain the specific rate of OTCase synthesis, therefore, counts incorporated into OTCase CRM were divided by counts incorporated into bulk protein during the same pulse. It was apparent (Fig. 2) that OTCase synthesis decreased to a low level by about 2 h after the peak of enzyme activity. The results indicated that measurement of OTCase protein by immunoprecipitation of OTCase CRM from cells labeled with [^{3}H]leucine should not be seriously interfered with by continued OTCase synthesis during the early part of inactivation. The rate of OTCase degradation from 17 h onward could not be accurately measured by this procedure, since a significant amount of OTCase synthesis occurred during that time.

Radiolabeling with [^{3}H]leucine and immunoprecipitation was used as outlined above to compare the rates of OTCase inactivation and loss of OTCase CRM in strain 168 before sporulation in glucose-nutrient broth medium (Fig. 3). The loss of activity was closely paralleled by the loss of CRM; no inactive CRM was accumulated during inactivation. Only one peak of radioactive protein, which migrated in the position corresponding to the OTCase subunit (M_r = 44,000), was detected on electrophoretic analy-

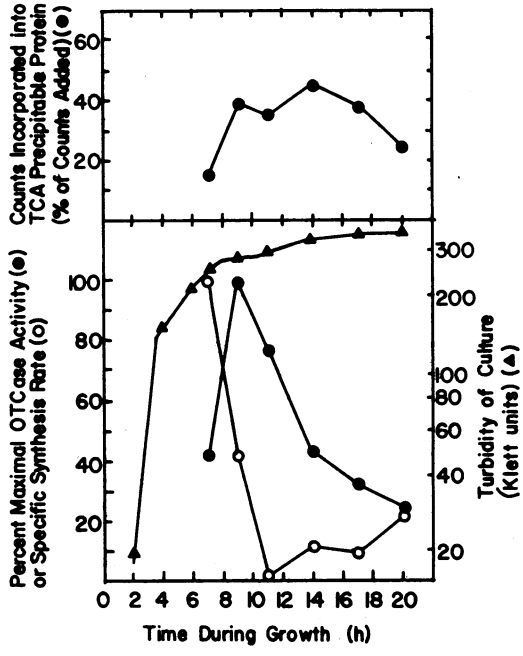

FIG. 2. Rate of OTCase and bulk protein synthesis during OTCase inactivation in strain 168. Samples (32 ml) of a 500-ml culture growing in 0.1% glucose-nutrient broth medium were labeled for 20 min with 10 μCi of L-[4,5-^{3}H]leucine per ml at the times shown (○, ●). After 20 min the cells were harvested, washed, extracted, and assayed for OTCase. Samples of the extracts were treated with anti-OTCase antibodies, and the immunoprecipitates were analyzed by electrophoresis on SDS-polyacrylamide tube gels. The radioactivity incorporated into OTCase at the 100% synthesis point was 25,000 cpm in the sample analyzed. Incorporation of [^{3}H]leucine into trichloracetic acid (TCA)-precipitable protein (●) was determined with another sample (top). Specific synthesis rate of OTCase (○) was obtained by dividing the disintegrations per minute incorporated into OTCase by those incorporated into protein and normalizing to 100% at the fastest rate. Maximal (100%) OTCase activity was 117 mU per ml of culture.

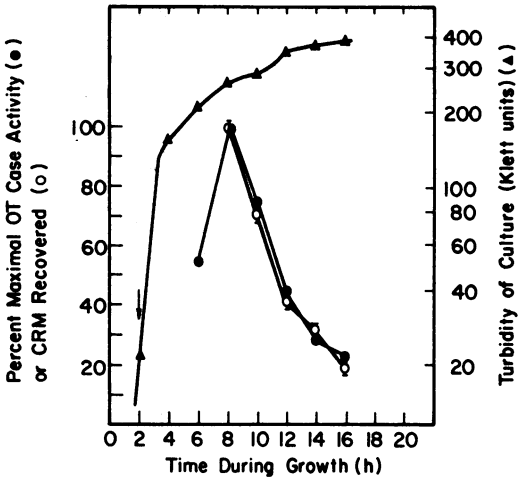

FIG. 3. OTCase inactivation and degradation in strain 168. The cells were grown in 500 ml of 0.1% glucose-nutrient broth. L-[4,5-^{3}H]leucine (4 μCi per ml of culture) was added at 2 h (arrow). OTCase activity (●) and OTCase CRM (○) were determined as described in the text. The radioactivity in OTCase CRM at the 100% point (8 h) was 6,000 cpm in the sample analyzed, and the OTCase activity at this point was 165 mU per ml of culture.

sis of the immunoprecipitates. A double immunoprecipitation experiment, using goat antibodies against rabbit immunoglobulin G in addition to the anti-OTCase antibodies, gave a similar result. Given the ability of the anti-OTCase antibodies to detect denatured and fragmented forms of OTCase, the results indicate that OTCase inactivation in vivo involves, or is rapidly followed by, extensive degradation of the protein.

Degradation of OTCase in vitro. Paulus (Ph.D. thesis, University of Illinois, Urbana, 1979) observed that OTCase activity was not lost when crude extracts of *B. subtilis* cells were incubated at 30°C for several hours. However, these extracts did not contain Ca^{2+} ions, which are required for ISP activity and stability (17–19). Extracts from cells engaged in OTCase inactivation (harvested at 12 h; see Fig. 1) were prepared in 50 mM Bicine-NaOH (pH 8.3) containing 2 mM $CaCl_2$. These were incubated at 30°C and assayed at various times. No loss of OTCase activity was seen over 5 h. Addition of 3 mM Mg-ATP to the incubation system had no effect. Extracts from cells grown in the presence of [^{3}H]leucine were incubated under similar conditions, and immunoprecipitates of the OTCase were analyzed by SDS-polyacrylamide electrophoresis. There was no detectable change in either the amount or the molecular weight of the CRM during the incubation. Thus, the enzyme was probably not degraded during the incubation, although removal of a small peptide could not have been detected.

Since degradation of OTCase in vitro may well have been interfered with by the many other components in crude extracts, we examined the possible degradation of purified OTCase by highly purified ISP from *B. subtilis*. This protease was chosen because OTCase inactivation had been shown to proceed fourfold more slowly in a mutant lacking it (Fig. 1; 13). Purified ISP (0.15 mg/ml) and OTCase (0.2 mg/ml) were incubated together in 50 mM Tris-hydrochloride, pH 7.5 (at 30°C), containing 2 mM $CaCl_2$ at 30°C. No loss of OTCase activity was found during the 4 h incubation. To determine whether any fragmentation of OTCase occurred under these conditions, samples were removed at various times, quenched by the addition of 2 mM phenylmethylsulfonyl fluoride, and electrophoresed on a 10% SDS-polyacrylamide slab gel. No lower-molecular-weight species were generated over the course of 4 h of incubation with ISP. This was true even after extended incubations (4 to 5 h) in the presence of high concentrations of ISP (0.2 mg/ml) and in spite of the fact that OTCase could be fragmented by much lower concentrations of trypsin or chymotrypsin in a much shorter time (data not shown). The following potential effectors were evaluated under similar conditions at 3 mM final concentrations and found to be without effect on degradation: 2-mercaptoethanol, ornithine, carbamyl phosphate, phosphonacetyl-L-ornithine (6), citrulline, arginine, Mg-ATP, Mg-ppGpp, and Mg-pppGpp. The ability of the purified ISP to degrade purified *B. subtilis* aspartate transcarbamylase and glutamine phosphoribosylpyrophosphate amidotransferase under comparable conditions was demonstrated, and the ISP was highly active in the degradation of azocollagen and azocasein (Neway, Ph.D. thesis).

When OTCase was incubated with 1% SDS in 50 mM Tris-hydrochloride, pH 7.5 (at 30°C), and 2 mM $CaCl_2$ at 30°C for 10 min before the addition of ISP, the enzyme was extensively degraded within 5 to 10 min. Of the various effectors listed in the previous paragraph, carbamyl phosphate and phosphonacetyl-L-aspartate, strongly inhibited OTCase degradation in the presence of SDS. These findings indicate that SDS bound to OTCase, bringing about conformational changes that rendered it susceptible to degradation by ISP. The substrate and substrate analog presumably acted to stabilize the OTCase against such partial denaturation. The degradation of OTCase by ISP in the presence of SDS required Ca^{2+} ions and was stimulated further by addition of Mg^{2+} ions.

Further characterization of OTCase inactivation in the ISP-deficient mutant. The failure to

observe degradation of purified native OTCase by purified ISP led us to examine further the properties of the ISP-deficient mutant, strain S-87, and the inactivation of OTCase in this strain. We first tested the possibility that strain S-87 was not truly deficient in ISP. The maximal azocollagenase activity in the mutant was about 20% that of its parent, strain 168 (Fig. 1). This was shown to be true throughout the pH range from 5.5 to 11, using both azocollagen and azocasein as protease substrates. Thus, the deficiency in strain S-87 was not the result of a shift in pH optimum or substrate specificity.

Purification of ISP from extracts of strain S-87 by the same procedure as used from the parent strain was attempted, following activity with azocollagen as the substrate. The low level of protease activity from strain S-87 behaved the same as that from strain 168 during extract preparation and ammonium sulfate fractionation. The dialyzed ammonium sulfate fraction was applied to a DEAE-cellulose (DE-52) column and washed with 300 mM Tris-hydrochloride buffer as usual. Under these conditions, the only protease activity detected was washed through. When a 300 to 700 mM Tris-hydrochloride (pH 8.5) gradient was applied, no further activity was eluted. In contrast, ISP from strain 168 was eluted from the DE-52 column only when the Tris-hydrochloride concentration in the gradient reached about 500 mM. Only a small amount of collagenase activity from strain 168 washed through the column with 300 mM Tris-hydrochloride. The results suggest that normal ISP was completely absent from cells of strain S-87. The collagenase activity that washed through the DE-52 column was probably catalyzed by other proteolytic enzymes.

Degradation of OTCase during its inactivation in cells of strain S-87 was examined by using the radiolabeling and immunoprecipitation technique used with strain 168, as in Fig. 3. Both OTCase activity and CRM were lost slowly from strain S-87, as expected, but tritium label in the CRM appeared to be lost more rapidly than activity (Fig. 4). Such a peculiar result might have been seen if the specific radioactivity of the enzyme did not remain constant during inactivation/degradation. This could occur if synthesis of OTCase continued in strain S-87 (unlike strain 168) during inactivation and if the specific radioactivity of the precursor leucine was lower during degradation than it was earlier when OTCase was accumulating in the cells. To minimize this possibility, the experiment shown in Fig. 4 was repeated but with 75 μg of leucine per ml added to the medium to ensure that leucine of a constant specific radioactivity was present in the medium throughout growth. Once again, the tritium label in the OTCase CRM was lost at a

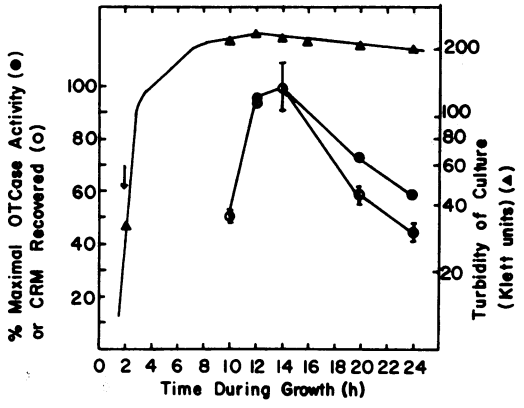

FIG. 4. OTCase inactivation and degradation in strain S-87. Methods were as in the legend to Fig. 3, except that 6 μCi of [^{3}H]leucine per ml of culture was added. The radioactivity in OTCase CRM at the 100% point (14 h) was 4,200 cpm in the sample analyzed, and the OTCase activity at this point was 93 mU/ml.

slightly faster rate than activity. Since the permeability of *Bacillus* cells to amino acids decreases in stationary phase (2), artifacts of labeling during continued OTCase synthesis could not be ruled out. It thus became necessary to examine the rate of OTCase synthesis in strain S-87, as had been done previously with strain 168.

Continued synthesis of OTCase during its inactivation in strain S-87 was determined by pulse-labeling with [^{3}H]leucine and immunoprecipitation of the newly synthesized OTCase (see Fig. 2). In the mutant strain the synthesis of OTCase continued at a substantial rate for an extended time during the period of apparently slow OTCase inactivation (Fig. 5). This was in contrast to OTCase synthesis in strain 168, which reached a low level shortly after inactivation had begun (see Fig. 2). OTCase synthesis also increased to a significant level at very late times in strain 168 (after 17 h), but did not reach the level found before inactivation began or the high level seen during inactivation in strain S-87. These results suggested that the slow rate of disappearance of OTCase from cells of strain S-87 was not a result of slower inactivation, but resulted instead from the inability of this mutant to shut off OTCase synthesis.

In a final attempt to minimize the effects of continued OTCase synthesis on our radioimmunochemical determination of the rate of loss of OTCase CRM, the experiments of Fig. 2 (strain 168) and 4 (strain S-87) were repeated exactly as before, except that at the onset of OTCase inactivation (10 h for strain 168; 14 h for strain S-87) a large "chase" of 1,000 μg of nonradioactive leucine per ml of culture was

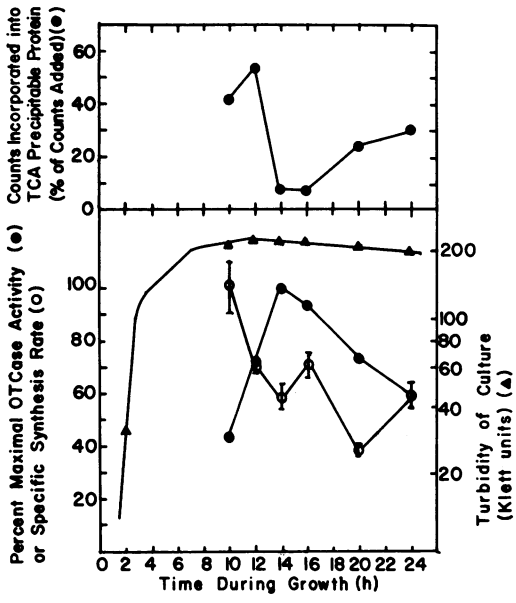

FIG. 5. Rate of OTCase and bulk protein synthesis during OTCase inactivation in strain S-87. The experiment was conducted and the data were analzyed as in the legend to Fig. 2. Samples of 21 ml were labeled for 20 min with 14 μCi of [^{3}H]leucine per ml. The radioactivity incorporated into OTCase CRM at the 100% point (10 h) was 100,000 cpm in the sample analyzed, and the OTCase activity at this point was 120 mU per ml of culture. TCA, Trichloroacetic acid.

added. If the cells were freely permeable to leucine, such a chase should have prevented further incorporation of [^{3}H]leucine into OTCase, so that the loss of radioactivity from immunoprecipitate should reflect only loss of previously synthesized enzyme. Since the permeability of stationary cells to amino acids may be limited (2), the rates of decay of prelabeled OTCase CRM seen in such experiments represented a minimal rate; faster rates could not be excluded. The decay of ^{3}H-labeled OTCase CRM in strain 168 proceeded under these conditions with a half-life of about 2.8 h. This was slightly, but significantly, faster than the loss of OTCase activity and the rate of decay of both activity and ^{3}H-labeled CRM seen without the chase in Fig. 2 (half-life, about 3.6 h). This result suggests that, even in strain 168, a small amount of residual OTCase synthesis was detectable during OTCase degradation. The results with strain S-87 were more dramatic. The loss of OTCase activity proceeded at essentially the same rate as in Fig. 4 (half-life, roughly 12 h), but the ^{3}H-labeled OTCase CRM was lost with a half-life of about 3.8 h. The differences confirm the conclusion reached above that OTCase synthesis continued at a very substantial rate during its inactivation in strain S-87. The degradation of OTCase CRM in strain S-87 was not quite so fast as in the parent strain, but the major difference in the OTCase content of stationary cells of these two strains results from a defect in the control of OTCase synthesis, not from an inability to degrade the protein.

DISCUSSION

A number of enzyme activities disappear from *B. subtilis* cells during the stationary phase of growth. Aspartate transcarbamylase (4, 9, 22), glutamine phosphoribosylpyrophosphate amidotransferase (15, 21), and pyrimidine-repressible carbamyl phosphate synthetase (13) all begin to be inactivated immediately after the end of exponential growth. OTCase, on the other hand, is first inactivated 4 to 6 h after the end of exponential growth on glucose-nutrient broth, which is a period of extensive bulk protein turnover (10). The evidence presented here indicates that inactivation of OTCase, like that of the transcarbamylase and the amidotransferase, either involved or was rapidly followed by extensive proteolytic degradation. This conclusion follows from the simultaneous disappearance of OTCase activity and CRM during inactivation and the demonstration that the antibodies used were capable of detecting denatured and fragmented forms of the enzyme. As has been pointed out elsewhere (9), such evidence can never be made logically conclusive, because one cannot be certain that a particular denatured species of OTCase formed in vivo would have been detected by the immunochemical techniques used. Nonetheless, we believe that it is unlikely that accumulation of unproteolyzed inactive OTCase would have escaped detection, unless such material was very insoluble and easily sedimented. Insoluble aggregates are found during massive accumulations of abnormal proteins, but have not been detected during degradation of normal proteins (14).

Unlike the inactivation of aspartate transcarbamylase in *B. subtilis* (22), the inactivation of OTCase was not inhibited by interfering with the generation of metabolic energy by addition of fluoroacetate. Inactivation was prevented by chloramphenicol, if the antibiotic was added before inactivation began. Presumably some protein must be synthesized in stationary cells to allow normal inactivation, because the antibiotic did not inhibit degradation once it was under way. This is another difference between the mechanisms of aspartate transcarbamylase and OTCase inactivation. Degradation of aspartate transcarbamylase is not prevented by addition of chloramphenicol at any time (9). The degradation (but not inactivation) of glutamine phosphoribosylpyrophosphate amidotransferase, on the other hand, can also be blocked by early

addition of chloramphenicol or other inhibitors of protein synthesis (15), so it is possible that the degradation of OTCase and that of amidotransferase share some mechanistic elements.

There is no evidence that ISP is involved in the inactivation/degradation of OTCase in vivo, and there is considerable evidence against this idea. Purified ISP failed to degrade OTCase in vitro, except when OTCase was denatured with SDS. It is possible that the degradation in vivo requires some regulatory factor which mimics the denaturation of OTCase and renders it susceptible to proteolysis, but none of the metabolites we tested were able to do this. Furthermore, such a regulatory factor failed to promote degradation in crude extracts containing OTCase and ISP. It should also be noted that addition of chloramphenicol to *B. subtilis* 168 cells at 8 h prevented OTCase degradation, even though such cells contained half their maximal level of ISP. The involvement of ISP in OTCase degradation was originally suggested by the slow rate of loss of OTCase from the ISP-deficient mutant strain S-87. The demonstration that OTCase synthesis was not shut off normally in this strain, however, provided an alternative interpretation. When care was taken to minimize the effects of continued OTCase synthesis on the measurement of OTCase degradation, OTCase was shown to be degraded nearly as rapidly in strain S-87 as in the parent strain (half-lives, 3.8 and 2.8 h, respectively).

The precise nature of the defect in strain S-87 is unknown. The strain is pleiotropic, because it fails to produce both ISP and bacillopeptidase F (5). The failure of the mutant to regulate OTCase synthesis normally leads us to speculate that it may be defective in the regulation of transcription in the stationary phase. An alternative possibility is that OTCase synthesis might continue in strain S-87 because protein turnover is deficient in this mutant, which could lead to failure to accumulate a level of intracellular arginine that normally represses OTCase late in the stationary phase. We tested this possibility by adding 100 μg of arginine per ml of culture to stationary cultures of S-87 (data not shown). Not only did arginine addition fail to repress OTCase synthesis, but it resulted in increased OTCase synthesis, as it had been shown to do in the early stationary phase of the parent strain (11). Furthermore, the OTCase activity then declined as slowly as in a culture to which no arginine was added. These results show that OTCase synthesis does not continue in stationary S-87 cells because of failure of arginine to repress the enzyme.

The results of this study point to the need for extreme caution in the interpretation of experiments that use protease-deficient mutants for the study of enzyme degradation, unless the mutation is known to reside in the structural gene for the protease. Such mutants are not available in *B. subtilis* and would be of great value (10). In this context, a *B. subtilis* mutant strain (TS-15), which was reported by Kerjan et al. (8) to be temperature sensitive in ISP, did not prove useful, because in our hands it produced intracellular protease, sporulated, and inactivated OTCase at the restrictive temperature, although it did so 6 to 8 h later than its parent strain (Neway, Ph.D. thesis).

ACKNOWLEDGMENT

These studies were supported by Public Health Service grant AI-11121 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Anderson, L. E., and W. O. McClure.** 1973. An improved scintillation cocktail of high-solubilizing power. Anal. Biochem. **51**:173–179.
2. **Bernlohr, R. W.** 1965. Role of amino acids in sporulation, p. 75–87. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
3. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
4. **Deutscher, M. P., and A. Kornberg.** 1968. Biochemical studies of bacterial sporulation and germination. VII. Patterns of enzyme development during growth and sporulation of *Bacillus subtilis*. J. Biol. Chem. **243**:4653–4660.
5. **Hageman, J. H., and B. C. Carlton.** 1973. Effects of mutational loss of specific intracellular proteases on the sporulation of *Bacillus subtilis*. J. Bacteriol. **114**:612–617.
6. **Hoogenraad, N. J.** 1978. Synthesis and properties of *N*-(phosphonacetyl)-L-ornithine. A transition-state analog inhibitor of ornithine transcarbamylase. Arch. Biochem. Biophys. **188**:137–144.
7. **Jentoff, N., and D. G. Dearborn.** 1979. Labeling of proteins by reductive methylation using cyanoborohydride. J. Biol. Chem. **254**:4359–4365.
8. **Kerjan, P., E. Keryer, and J. Szulmajster.** 1979. Characterization of thermosensitive sporulation mutant of *Bacillus subtilis* affected in the structural gene of an intracellular protease. Eur. J. Biochem. **98**:353–362.
9. **Maurizi, M. R., J. S. Brabson, and R. L. Switzer.** 1978. Immunochemical studies of the inactivation of aspartate transcarbamylase by stationary phase *Bacillus subtilis* cells. J. Biol. Chem. **253**:5585–5593.
10. **Maurizi, M. R., and R. L. Switzer.** 1980. Proteolysis in bacterial sporulation. Curr. Top. Cell Regul. **16**:163–224.
11. **Neway, J. O., and R. L. Switzer.** 1983. Purification, characterization, and physiological function of *Bacillus subtilis* ornithine transcarbamylase. J. Bacteriol. **155**:512–521.
12. **Ozotova, L. S., D. I. Gorodetskii, V. V. Yanonis, L. A. Baratova, L. P. Belyanova, E. A. Timokhina, A. Y. Strongin, and V. M. Stepanov.** 1978. Isolation of an intracellular serine protease from *Bacillus subtilis* by affinity chromatography on gramacidin-S-Sepharose. Bioorgan. Khim. **4**:397–403.
13. **Paulus, T. J., and R. L. Switzer.** 1979. Synthesis and inactivation of carbamyl phosphate synthetase isozymes of *Bacillus subtilis* during growth and sporulation. J. Bacteriol. **140**:769–773.
14. **Prouty, W. F., M. J. Karnovsky, and A. L. Goldberg.** 1975. Degradation of abnormal proteins in *Escherichia*

coli. Formation of protein inclusions in cells exposed to amino acid analogs. J. Biol. Chem. **250**:1112–1122.

15. **Ruppen, M. E., and R. L. Switzer.** 1983. Degradation of *Bacillus subtilis* glutamine phosphoribosylpyrophosphate amidotransferase *in vivo*. J. Biol. Chem. **258**:2843–2851.
16. **Setlow, P., and G. Primus.** 1975. Protein metabolism during germination of *Bacillus megaterium* spores. I. Protein synthesis and amino acid metabolism. J. Biol. Chem. **250**:623–630.
17. **Stepanov, V. M., A. Y. Strongin, L. S. Izotova, Z. T. Abramov, L. A. Lyublinskaya, L. M. Ermakova, L. A. Baratova, and L. P. Belyanova.** 1977. Intracellular serine protease from *Bacillus subtilis*. Structural comparison with the extracellular serine proteases-subtilisins. Biochem. Biophys. Res. Commun. **77**:298–305.
18. **Strongin, A. Y., D. I. Gorodetsky, I. A. Kuznetsova, V. V. Yanonis, Z. T. Abramov, L. P. Belyanova, L. A. Baratova, and V. M. Stepanov.** 1979. Intracellular serine proteinase of *Bacillus subtilis* strain Marburg 168: comparison with the homologous enzyme from *Bacillus subtilis* strain A-50. Biochem. J. **179**:333–339.
19. **Strongin, A. Y., L. S. Izotova, Z. T. Abramov, D. I. Gorodetsky, L. M. Ermakova, L. A. Baratova, L. P. Belyanova, and V. M. Stepanov.** 1978. Intracellular serine protease of *Bacillus subtilis*: sequence homology with extracellular subtilisins. J. Bacteriol. **133**:1401–1411.
20. **Switzer, R. L.** 1977. The inactivation of microbial enzymes *in vivo*. Annu. Rev. Microbiol. **31**:135–157.
21. **Turnbough, C. L., and R. L. Switzer.** 1975. Oxygen-dependent inactivation of phosphoribosylpyrophosphate amidotransferase in stationary-phase cultures of *Bacillus subtilis*. J. Bacteriol. **121**:108–114.
22. **Waindle, L. M., and R. L. Switzer.** 1973. Inactivation of aspartic transcarbamylase in sporulating *Bacillus subtilis*: demonstration of a requirement for metabolic energy. J. Bacteriol. **114**:517–527.
23. **Yousten, A. A., and R. S. Hanson.** 1972. Sporulation of tricarboxylic acid cycle mutants of *Bacillus subtilis*. J. Bacteriol. **109**:886–894.