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Purification, Characterization, and Physiological Function of *Bacillus subtilis* Ornithine Transcarbamylase

JUSTIN O. NEWAY† AND ROBERT L. SWITZER*
Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

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A procedure was developed for purification of ornithine transcarbamylase (OTCase) to near homogeneity from *Bacillus subtilis* 168. The purified native enzyme existed as a mixture of dimeric, tetrameric, and hexameric forms, but was converted to the dimer in the presence of 2-mercaptoethanol. The molecular weight of the subunit was 44,000. Some general kinetic properties of the enzyme were described. OTCase was repressed by arginine in growing *B. subtilis* cells, but the enzyme was induced by arginine at the end of exponential growth. Specific antibodies against the purified OTCase were used to show that the same enzyme was produced under all conditions. These results and studies of a mutant lacking OTCase demonstrated that *B. subtilis* produced only a single OTCase. OTCase was clearly required for arginine biosynthesis, but the physiological function of OTCase induction by arginine was obscure. OTCase was not induced by, or required for, growth on arginine as a carbon and nitrogen source. Absence of OTCase in a mutant did not alter the yield or arginine content of its spores in comparison to a strain containing OTCase.

Ornithine transcarbamylase (OTCase; EC 2.1.3.3) catalyzes an essential step in arginine biosynthesis. In those organisms that possess the arginine deiminase pathway OTCase also plays a catabolic role, whereas OTCase activity is not required for catabolism of arginine via the arginase pathway (Fig. 1; for review, see reference 1). Organisms that use OTCase for both biosynthetic and catabolic functions, such as *Pseudomonas aeruginosa*, generally elaborate two OTCases which are differentially regulated (17, 30). *Bacillus subtilis* produces a biosynthetic, arginine-repressible OTCase (11). A mutant, strain BR85, that lacks OTCase activity is an arginine auxotroph (19). *B. subtilis* is one of the relatively few bacterial species that degrade arginine by the arginase pathway (7). Whether this organism can also degrade arginine via the arginine deiminase pathway is uncertain. Arginine deiminase has not been detected in *B. subtilis* (17), but carbamate kinase has been reported to be present (12).

The complex regulation of OTCase activity in *B. subtilis* suggests that the enzyme may play a catabolic role, in addition to its well-established biosynthetic role (17, 24; T. J. Paulus, Ph.D. thesis, University of Illinois, Urbana, 1979). OTCase activity is fully repressed by arginine in exponentially growing cells, but the enzyme is induced by arginine in the early stationary phase of growth to levels three- to fourfold higher than in cells grown without arginine. No such induction occurs in the arginine auxotroph BR85 (Paulus, Ph.D. thesis), but there is otherwise no indication whether the same or different OTCases are produced under biosynthetic and arginine-inducing conditions. A closely related organism, *B. licheniformis*, possesses both the arginase and the arginine deiminase pathways (4, 5) and has been reported to produce separate biosynthetic and catabolic OTCases, whose regulation resembles that found with *B. subtilis* (4, 5, 16).

As part of a study of the degradative inactivation of OTCase in sporulating *B. subtilis* cells (23, 25), we needed highly purified OTCase to prepare monospecific antibodies directed against it. In this paper we report a procedure for purification of *B. subtilis* OTCase to near homogeneity and a general physical and kinetic characterization of the enzyme. Experiments indicating that *B. subtilis* produces only a single OTCase under all growth conditions are presented, but the physiological function of the induction of OTCase by arginine in stationary cells remains unclear.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strain 168 (*trpC2*), obtained from J. H. Hageman, was used in this study unless otherwise stated. Strain BR85 (*trpC2 argC4*)

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

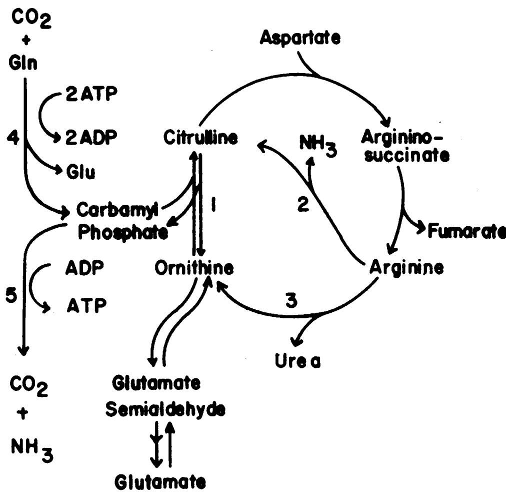

FIG. 1. Pathways of arginine metabolism in microorganisms. Enzymes discussed in the text are: 1, OTCase; 2, arginine deiminase; 3, arginase; 4, carbamyl phosphate synthetase; 5, carbamate kinase.

and its parent strain JH862 (*trpC2*) were provided by J. A. Hoch.

Media and culture methods. A buffered minimal medium containing the salts mixture described by Anagnostopoulos and Spizizen (2), 0.1% glucose, and 50 μg each of 19 amino acids (Ala, Asp, Asn, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, but no arginine) per ml was used, unless otherwise stated. Cells used for the large-scale isolation of OTCase were grown in a 200-liter fermentor on the same medium, except that the 19 amino acids were added at 30 μg/ml each and 150 μg of arginine per ml was included. Cells from the fermentor were harvested within 1 h after the end of exponential growth, when the level of OTCase reached its peak. When strains BR85 and JH862 were grown on arginine as the sole source of carbon and nitrogen, the same salts mixture (2) was used, except that $(NH_4)_2SO_4$ was replaced by an equimolar amount of Na_2SO_4, glucose was replaced by 0.4% arginine, and one-tenth the trace minerals mixture used in supplemented nutrient broth cultures (8) was included. No amino acids were added to the medium except arginine and 50 μg of tryptophan per ml. The techniques used for growing such cultures with poor aeration were the same as described by Broman et al. (5). All cultures were incubated at 37°C with vigorous aeration, except when noted otherwise.

Cell extracts. Cells were harvested by centrifuging at 20,000 × *g* and 4°C for 5 min. Cell pellets were washed by resuspension first in 25 mM Bicine-NaOH (pH 8.3), then in 25 mM Bicine-NaOH (pH 8.3) containing 1 M KCl, and finally in 25 mM Bicine-NaOH (pH 8.3) containing 0.1 M KCl. Cells were sedimented between resuspensions by centrifugation as above. Cell pellets were stored at −20°C.

Cell extracts were prepared by suspending frozen cell pellets in 50 mM Bicine-NaOH (pH 8.3) containing 2 mM $CaCl_2$ at 10- or 20-fold concentration over the culture. Cells were disrupted by sonication with a Branson Sonifier model W185 (Heat Systems Ultrasonics, Inc.) at a power setting of 4, with the output tuned to 70 W and with intermittent cooling in an ice bath. Duration of sonication was determined by examination of cell breakage with a microscope. Extracts were clarified by centrifugation at 20,000 × *g* and 4°C for 20 min.

Assays. OTCase was assayed by measuring the rate of citrulline formation in the presence of ornithine and carbamyl phosphate. The reaction mixture contained 0.4 ml of 250 mM Bicine-NaOH (pH 8.3), 0.1 ml of 100 mM ornithine, 0.1 ml of freshly dissolved 100 mM carbamyl phosphate (dilithium salt; Sigma Chemical Co.), and glass-distilled water and cell extract to bring the final volume to 1 ml. When required, the enzyme was diluted with 250 mM Bicine-NaOH, pH 8.3. Reactions were initiated by the addition of cell extract. Before this addition, the reaction mixture was held on ice to prevent the spontaneous formation of citrulline. It was placed in a 30°C water bath immediately upon addition of extract and held for 15 to 20 min before being quenched with 1 ml of 5% (vol/vol) perchloric acid. Blanks were analyzed in parallel, using distilled water instead of cell extract, or were incubated with cell extract and without carbamyl phosphate (added after quenching) if correction for ureido compounds present in the extract was required. The amount of citrulline present in a 0.2-ml sample of the quenched assay mixture was measured by the method of Prescott and Jones (27) as modified by Shindler and Prescott (28). One unit of OTCase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of citrulline per min at 30°C in 100 mM Bicine-NaOH at pH 8.3.

To locate OTCase activity in nondenaturing gels, gel slices (1 mm thick) were each placed individually in 0.4 ml of 250 mM Bicine-NaOH (pH 8.3; 30°C)–0.1 ml of 100 mM ornithine–0.36 ml of glass-distilled water. The slices were crushed with a glass rod, and the mixture was allowed to incubate at 30°C for 1 h or at 4°C overnight. The reaction was initiated by the addition of 0.1 ml of 100 mM carbamyl phosphate and incubated at 30°C. The assays were quenched, and the amount of citrulline was measured as described previously.

Protein was measured by the procedure of Lowry et al. (18), with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis. Vertical slab gel electrophoresis was performed in an apparatus similar to the one described by Studier (31), using the discontinuous buffer system containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) described by Laemmli (15). Slabs contained either 10% (wt/vol) acrylamide monomer or a gradient of 15% (wt/vol; bottom) to 7.5% (wt/vol; top) acrylamide monomer. Electrophoretic separation of proteins under nondenaturing conditions in 7% polyacrylamide gels was accomplished by the method of Davis (6). Gels were stained with Coomassie blue.

Immunochemical methods. Antibody against purified OTCase was elicited in a female New Zealand white rabbit. The primary injection was a 50% (vol/vol) emulsion of complete Freund adjuvant with 1 mg of highly purified OTCase in 50 mM Tris-hydrochloride, pH 7.5. Half of this emulsion was injected into each hind leg muscle. Two booster injections, each containing 1 mg of OTCase from the same OTCase solution as used for the primary injection, were made as 50% (vol/vol) emulsions of incomplete Freund adjuvant. These

were administered 1 and 2 weeks after the initial injection, each in four locations over the shoulders. Serum was collected over a period of 1 month by bleeding from the ear vein.

Separation of serum and isolation of immunoglobulins were performed by standard procedures (32). Lipids were removed by treatment with dextran sulfate, and the immunoglobulins were purified by precipitating twice with ammonium sulfate (40% saturation). The immunoglobulin was separated by chromatography on DEAE-cellulose.

Immunoprecipitation of OTCase from cell extracts. Cell cultures were labeled by sterile addition of L-[4,5-^{3}H]leucine (60 Ci/mmol) as an aqueous solution in 2% (vol/vol) ethanol. Thirty- or 20-ml samples were harvested and washed three times as above. Frozen cell pellets were resuspended at 10- or 20-fold concentration over the original culture in Bicine-NaOH buffer (pH 8.3) containing 2 mM sodium EDTA and 1 mM freshly added phenylmethylsulfonyl fluoride. Phenylmethylsulfonyl fluoride was added from a 50 mM ethanol solution and gave a final concentration of ethanol in the extract of 2% (vol/vol). Cells were broken by sonication as described above. Extracts were centrifuged at either 20,000 × *g* for 20 min or 100,000 × *g* for 1 h at 4°C as stated and were used immediately for OTCase assays and immunoprecipitation without freezing.

Incubation mixtures for immunoprecipitation contained 0.6 or 0.9 ml of centrifuged cell extract, 25 μl of 1% sodium azide, and 10 μl of a 1.7-mg/ml solution of purified OTCase in a 1.5-ml Microfuge tube. Sufficient anti-OTCase was added so that there was a threefold excess over the amount required to precipitate all purified OTCase added. Solutions were incubated overnight at 4°C. Precipitates were collected by centrifuging for 1 min in a Beckman model B Microfuge at top speed and were washed three times by resuspending with a Vortex mixer in 0.3 ml of 50 mM Tris-hydrochloride (pH 7.9) containing 1 M NaCl, 1% (vol/vol) Triton X-100, and 0.5 mM sodium EDTA. Washed immunoprecipitates were resuspended in 0.1 ml of SDS-gel sample buffer diluted 1:1 with 8 M urea. These were placed in a boiling-water bath for 5 to 7 min for complete dissolution and were subjected to electrophoresis in 10% polyacrylamide–SDS tube gels.

Polyacrylamide gels were sliced into 1-mm slices. Each slice was placed in a plastic Minivial, and 0.4 ml of 15% (vol/vol) H_2O_2 was added. The vials were then capped and incubated at 75°C for 4 to 5 h. When the gel slices had dissolved, the vials were allowed to cool to room temperature, and 5 ml of scintillation cocktail (3) was added. An external standard method was used to determine the extent of quenching in each vial. The total efficiency of counting was usually 22 ± 2%.

Determination of the amino acid content of spores. Spores of strain JH862 and mutant BR85 were prepared and analyzed for amino acids by the method of Nelson and Kornberg (21). The spores were obtained from cells grown for 24 h at 37°C on supplemented nutrient broth (8) containing 0.1% glucose. Free amino acids were extracted from spores by boiling the spore suspension (10 mg of dry spores per ml of water) for 30 min. After extraction, the suspension was centrifuged at 30,000 × *g* and 4°C for 10 min, and the supernatant was stored at −20°C until analyzed. For determination of total amino acid content, 1 ml of spore suspension was diluted 1:1 with 12 N HCl and hydrolyzed for 18 h at 110°C under vacuum. Spore debris was removed by centrifugation at 30,000 × *g* and 4°C for 10 min. Amino acids were determined with a Beckman model 119 CL amino acid analyzer.

Purification of OTCase. All procedures were conducted at 4°C unless otherwise stated.

(i) Crude extract. *B. subtilis* 168 cells were grown in a 200-liter fermentor as described above and harvested by centrifugation. The cell paste was resuspended in 50 mM Tris-hydrochloride buffer (pH 7.9, 4°C) containing 1 M KCl and 1 mM freshly added phenylmethylsulfonyl fluoride and sedimented by centrifugation for 0.5 h (25,000 × *g* at 4°C). The cells were then washed a second time by resuspending in the same buffer without KCl or phenylmethylsulfonyl fluoride and centrifuging under the same conditions. This gave a washed cell paste of approximately 600 g, which was frozen in liquid nitrogen and stored at −20°C until used. (It was later observed that the washing of the cells could be eliminated without significant effects on the subsequent purification procedure or purity of the final OTCase preparation.) Cell paste from a single fermentor growth was resuspended in 1 liter of Bicine-NaOH buffer (pH 8.3) containing 2 mM sodium EDTA and 1 mM freshly added phenylmethylsulfonyl fluoride. The slurry was passed three times through a Manton-Gaulin pressure mill at 5,000 to 6,000 lb/in^2, taking care not to allow the effluent temperature to rise above 45°C. After each pass through the mill, the slurry was allowed to flow through a stainless-steel coil immersed in an ice-water bath, so that its temperature was reduced to <10°C when it emerged. Streptomycin sulfate was added from a 30% (wt/vol) water solution to a final concentration of 1% (wt/vol), and the mixture was allowed to stand for 0.5 h.

(ii) Heat precipitation. The broken cell slurry was divided into two equal portions, and each portion was placed in a 2-liter Erlenmeyer flask and swirled vigorously in a 65°C water bath. When the temperature of its contents had reached 57°C, the flask was transferred to a 60°C water bath for 12 min of further incubation with occasional swirling. The heat-treated extract was then poured into metal centrifuge cans in a ice-water bath and allowed to cool. When the temperature of the extract reached approximately 10°C, the solids were sedimented by centrifugation at 25,000 × *g* for 45 min.

(iii) Ammonium sulfate precipitation. The supernatant fluid from the above centrifugation was stirred, and solid ammonium sulfate (special enzyme grade; Schwarz/Mann) was slowly added to 55% saturation (326 g/liter). The precipitate was removed by centrifugation at 25,000 × *g* for 45 min, and the supernatant was brought to 75% ammonium sulfate saturation, using solid ammonium sulfate (127 g/liter). This second precipitate was collected by centrifugation at 25,000 × *g* for 45 min, dissolved in 85 ml of 15 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium EDTA and 1 mM 2-mercaptoethanol, and dialyzed overnight against two changes (3.5 liters each) of the same buffer.

(iv) DEAE-cellulose chromatography. A column (3.9 by 30 cm) was packed with DEAE-cellulose (DE-52, Whatman) and equilibrated with 15 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium EDTA and 1 mM 2-mercaptoethanol. The dialyzed

ammonium sulfate fraction was pumped in and washed with 200 ml of the same buffer containing 100 mM KCl. A 1-liter linear gradient of 100 to 600 mM KCl in the same potassium phosphate buffer was then pumped through at a flow rate of 60 ml/h. Fractions, 10 ml, were collected and assayed for OTCase, which eluted as a single peak at a position corrresponding to a KCl concentration of 300 to 350 mM. When 2-mercaptoethanol was omitted from the buffer, OTCase activity eluted in two peaks, each of which had the same electrophoretic mobility in nondenaturing gels as the activity found in crude extracts. The fractions containing OTCase were pooled and dialyzed overnight against two changes (3.5 liters each) of 15 mM potassium phosphate buffer (pH 7.0 at room temperature) containing 2 mM sodium EDTA, but no 2-mercaptoethanol.

(v) Hydroxylapatite chromatography. A column (2.0 by 32 cm) was packed with extensively defined hydroxylapatite (Bio-Gel HTP; Bio-Rad Laboratories) and equilibrated with 15 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium EDTA. Mercaptoethanol was omitted from buffers used for this step, because OTCase eluted in two peaks when it was included. The dialyzed OTCase from the DEAE-cellulose step was loaded onto the column and eluted with a 500-ml linear gradient of 15 to 100 mM potassium phosphate (pH 7.0) containing 2 mM sodium EDTA. (Stepwise elution of OTCase with 30 mM potassium phosphate, pH 7.0, was also suitable for this step.) Fractions, 5 ml, were collected and assayed for OTCase, which eluted as a single broad peak at a position halfway through the gradient. Fractions containing OTCase were pooled and used directly for the next step without dialysis.

(vi) DEAE-Sephacel chromatography. A column 2.0 by 32 cm was packed with DEAE-Sephacel (Pharmacia Fine Chemicals, Inc.) and equilibrated with 15 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium EDTA and 1 mM 2-mercaptoethanol. The pool from the previous step was loaded and eluted with a 500-ml linear gradient of 0 to 400 mM KCl in the same buffer. Fractions, 5 ml, were collected and assayed for OTCase. Three peaks of protein eluted close together toward the end of the gradient. The large middle protein peak (corresponding to 300 mM KCl) contained the majority of the OTCase activity and very little contaminating protein. It was pooled and used directly for the next step without dialysis.

(vii) Bio-Gel A 1.5 chromatography. The OTCase pool from the previous step was brought to 75% saturation by slow addition of solid ammonium sulfate (467 g/liter). The precipitate was collected by centrifugation at 30,000 × *g* for 20 min and redissolved in 1 ml of 15 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium EDTA, but no 2-mercaptoethanol. A column (1.9 by 91 cm) was packed with Bio-Gel A 1.5 (100 to 200 mesh; Bio-Rad) and equilibrated with 15 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium EDTA. The redissolved ammonium sulfate pellet was loaded and eluted with the same buffer. Fractions, 5 ml, were collected and assayed. OTCase eluted at 152 ml, corresponding to a molecular weight of 260,000 ± 30,000. When 2-mercaptoethanol was included in the eluting buffer, OTCase activity eluted as a very diffuse peak at a volume greater than the column volume. Hence, 2-mercaptoethanol was not used in this step. Fractions containing OTCase activity were pooled, concentrated to a minimum of 1 to 2 mg/ml by vacuum dialysis, and stored frozen at −20°C. No loss of activity was seen after several months of storage with repeated freezing and thawing.

RESULTS

Purification of OTCase from *B. subtilis*. Table 1 summarizes the results of purifying OTCase from 600 g of frozen cell paste by the procedure described in Materials and Methods. The purity of the preparation was assessed at various stages by electrophoresis in polyacrylamide slab gels in the presence of SDS (Fig. 2). The final preparation was very nearly homogeneous, although traces of impurities could be detected on heavily loaded gels. The purity of this preparation was not increased by the use of several modifications of the purification procedure, repetition of the last three steps of the purification, or affinity chromatography on phosphonacetyl-L-ornithine conjugated to Sepharose (10).

Subunit and native molecular weight. SDS-polyacrylamide gel electrophoretic analysis of purified OTCase by the method of Neville (22) gave a subunit molecular weight of 44,000 ± 3,000. OTCase activity eluted from a Bio-Gel filtration column at a volume corresponding to a molecular weight of 260,000 when thiols were not present. This implied a hexameric state of aggregation for the native enzyme and was close to the molecular weight found by Issaly and Issaly (11). When thiols were present, OTCase apparently adsorbed to the agarose, because it

TABLE 1. Summary of OTCase purification from *B. subtilis* 168

Purification step	Total vol (ml)	Total protein (mg)	Total activity (U)	Yield (%)	Sp act (U/mg)	Fold purification
Crude extract	1,600	29,600	14,800	100	0.50	1
Heat	1,260	9,770	11,800	80	1.21	2.4
Ammonium sulfate	118	1,420	11,100	75	7.88	16
DEAE-cellulose	81	409	9,400	63	23	46
Hydroxylapatite	62	44	6,260	42	142	284
DEAE-Sephacel	24	16	3,820	26	235	470
Bio-Gel A 1.5	32	10	2,780	19	265	530

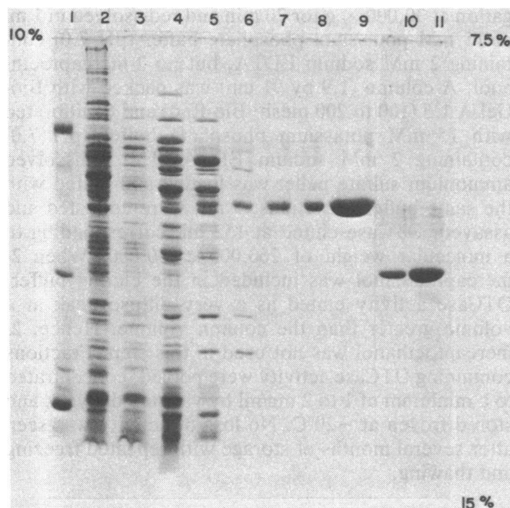

FIG. 2. SDS-polyacrylamide gel electrophoretic analysis of OTCase at various stages of purification. Lanes 1 to 9 were analyzed on 10% gels; lanes 10 and 11 were analyzed on a 7.5 to 15% polyacrylamide gradient gel. Samples analyzed were: lane 1, molecular weight standards, M_r = 67,000, 27,000, and 14,000; lane 2, crude extract; lane 3, heat step; lane 4, ammonium sulfate fraction; lane 5, DEAE-cellulose step; lane 6, hydroxylapatite step; lane 7, DEAE-Sephacel step; lanes 8 to 11, Bio-Gel A 1.5 step, 3, 15, 3, and 15 μg of OTCase, respectively.

was eluted at a volume greater than the total volume of the column.

When purified OTCase was analyzed by sucrose density gradient centrifugation, three peaks of activity were found (Fig. 3). Calibration of the gradients with catalase according to the method of Martin and Ames (20) yielded molecular weights of 99,000 ± 5,000, 167,000 ± 9,000, and 236,000 ± 20,000 for these peaks. These values corresponded to dimeric, tetrameric, and hexameric aggregation states. The relative amount of these three peaks varied from preparation to preparation of OTCase. However, when a single preparation of enzyme was examined, the pattern was not affected by protein concentration (from 0.2 to 6 mg/ml), prior incubation at 30°C for 1 h, or inclusion of carbamyl phosphate in the gradient. However, when 10 mM (or 3 mM) 2-mercaptoethanol was included in the sucrose gradients, only a single peak of activity (dimer) was found, even when a 30-fold protein concentration range was tested (Fig. 3).

When purified OTCase was analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions, multiple forms of the enzyme were also detected (Fig. 4, lane 1). When slices from an unstained gel were crushed, soaked in buffer, and assayed for OTCase, activity was found in each of the three major bands. (The slower migrating minor bands may also have been active, but these assays were less reliable.) When the three major bands were cut from an unstained gel, eluted overnight at 4°C, and electrophoresed under the conditions used previously, each migrated to its original position with little redistribution to positions of the other bands (Fig. 4, lanes 2, 3, and 4). We believe that only hexamer was detected by gel filtration on Bio-Gel because dimer and possibly tetramer adsorbed to the column matrix, resulting in their removal and losses in OTCase activity (see Table 1). When 1% 2-mercaptoethanol was included in the sample before electrophoresis, most of the protein and OTCase activity migrated in the position of the fastest moving band.

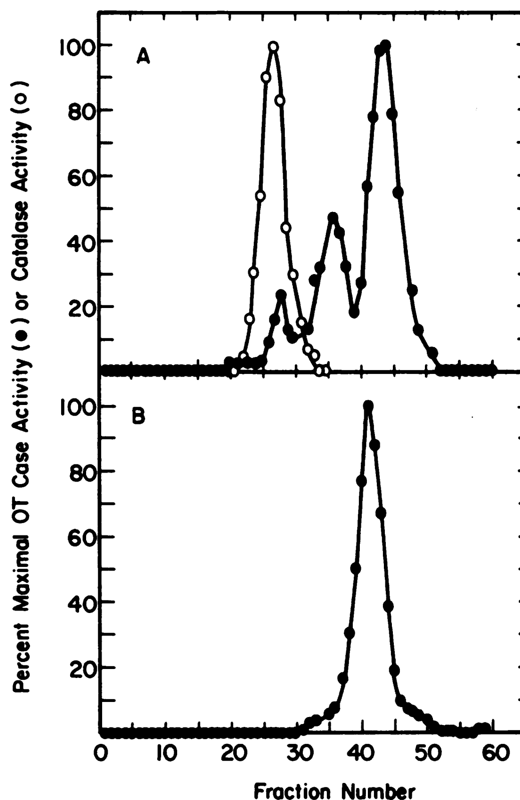

FIG. 3. Sedimentation of OTCase in sucrose gradients. Purified OTCase (20 μg) was centrifuged in a 5 to 20% (wt/vol) sucrose gradient containing 50 mM Tris-hydrochloride (pH 7.5) for 18 h at 100,000 × g and 4°C according to the procedure of Martin and Ames (20). The top of the gradient was to the right. Symbols: OTCase activity (●); catalase activity (○). (A) No 2-mercaptoethanol was included in the sample or gradient. (B) Separate sedimentation in which the sample and gradient contained 10 mM 2-mercaptoethanol. The thiol interfered with the catalase assay, so standardization relied on another gradient that contained catalase and no 2-mercaptoethanol.

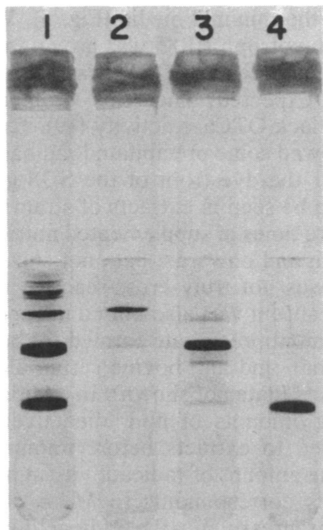

FIG. 4. Electrophoresis of purified OTCase on nondenaturing polyacrylamide gels lacking 2-mercaptoethanol. Purified OTCase (100 μg) was analyzed in two nondenaturing 7% polyacrylamide gels (6). One gel was stained for protein and is shown in lane 1. The three major bands were excised from the other gel, incubated separately in an equal volume of stacking gel buffer at 4°C overnight. These were then subjected to electrophoresis again and stained for protein. Lanes 2, 3, and 4 show the gels in which the upper, middle, and lower bands, respectively, were analyzed.

These results and those from the sucrose gradient studies described above suggested that OTCase existed in at least three states of aggregation when thiols were not present: hexamer, tetramer, and dimer. Electrophoresis in nondenaturing polyacrylamide gels of various percentages of monomer (22) indicated that the bands each had the same charge/mass ratio, which indicated that they were different aggregation states of the same protein. The relative ratio of aggregation states apparently depended on the protein concentration late in the purification procedure and was fixed when the enzyme preparation was originally concentrated under nonreducing conditions. On addition of thiols the higher aggregation states dissociated, and only the dimer could be found.

Kinetic properties of OTCase. The activity of *B. subtilis* OTCase was nearly constant from pH 7.5 to 10, although the optimum for activity and stability was at pH 8.3 in 100 mM Bicine-NaOH buffer. The activity dropped sharply to half-maximal at pH 7 and was negligible at pH 6. The use of 100 mM Tris-hydrochloride in the pH range from 7.5 to 8.5, as was done by other authors (11), gave only 40% as much activity as did 100 mM Bicine-NaOH, which was adopted routinely in our work.

The K_m values for carbamyl phosphate and ornithine were 0.9 and 5 mM, respectively, at pH 8.3 and 30°C. The concentrations of ornithine used in these studies were much lower than would be required to observe substrate inhibition, as described by Issaly and Issaly (11). The K_m for carbamyl phosphate was pH dependent and displayed a minimal value of 0.45 mM at pH 9.5.

Inhibition by phosphonacetyl-L-ornithine (prepared as described by Hoogenraad [9]) was competitive with respect to carbamyl phosphate and noncompetitive with respect to ornithine. This pattern of inhibition is consistent with (but does not establish fully) a kinetic mechanism commonly observed with transcarbamylases, namely, an ordered mechanism in which carbamyl phosphate binds first (26). The inhibition constant for phosphonacetyl-L-ornithine, determined at 10 mM ornithine and various carbamyl phosphate concentrations, was about 1 μM in the pH range from 7.5 to 9.0.

Immunochemical characterization of OTCase produced in the presence and absence of arginine. *B. subtilis* cells growing in the absence of added arginine produced OTCase at a maximal level of about 0.03 U per ml of culture. This activity was repressed to undetectable levels in exponentially growing cells by the presence of >50 μg of arginine per ml of culture (Paulus, Ph.D. thesis). On the other hand, when cells growing in the presence of high levels of arginine (100 to 200 μg/ml) entered the stationary phase, OTCase activity sharply increased from the repressed level to an "induced" level of about 0.10 U per ml of culture, even though this did not result in increased growth of the cells. The experiments in this section were designed to determine whether the OTCase produced by cells growing in the absence of arginine and by stationary cells induced by arginine were identical or different.

Two parallel cultures of *B. subtilis* strain 168 were grown in minimal medium containing 0.1% glucose. For one culture the medium contained all 18 amino acids (50 μg/ml) except leucine and arginine. The other culture was grown in medium with the same composition, except that arginine was present at 150 μg/ml. L-[4,5-^{3}H]leucine was added to both cultures at the start of growth to a final concentration of 2 μCi/ml. Cells were harvested when OTCase activity was maximal in each culture. Labeled OTCase was immunoprecipitated from extracts of these cultures. The immunoprecipitates were separated in SDS-polyacrylamide gels, and the radioactivity was counted. Only one peak of cross-reacting material (CRM) migrating in a position corresponding to an M_r of 44,000 could be found in extracts from either culture condition (Fig. 5). Extracts from strain 168 grown in L-[4,5-^{3}H]leu-

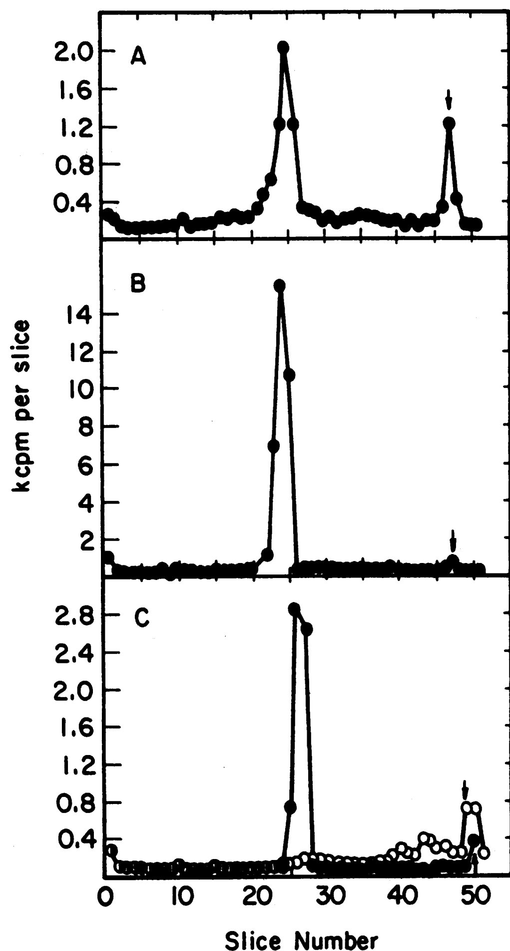

FIG. 5. Immunoprecipitation of OTCase from *B. subtilis* strains 168 and BR85 grown under various conditions. OTCase was immunoprecipitated from crude extracts prepared from cells grown as described below. Immunoprecipitates were analyzed by electrophoresis in 10% polyacrylamide-SDS tube gels. (A) immunoprecipitate from cells grown in minimal medium containing 0.1% glucose, 50 μg of each of 18 amino acids per ml (no leucine), 150 μg of arginine per ml, and L-[4,5-^{3}H]leucine (2 μCi/ml). Cells were harvested at the peak of OTCase activity (5 h). (B) Immunoprecipitate from cells grown in the same medium as in (A), except that no arginine was included. Cells were harvested during exponential growth. (C) Immunoprecipitate (●) from cells of strain 168 grown in supplemented nutrient broth (8) containing 0.1% glucose and 4 μCi of L-[4,5-^{3}H]leucine per ml. Cells were harvested at the peak of OTCase activity (10 h). Immunoprecipitate (○) from cells of strain BR85 grown in the same medium and harvested at the same time. Counts at the dye front (arrows) were not truly cross-reactive with OTCase (see text).

cine-containing glucose-nutrient broth (6 μCi/ml) also contained only one peak of OTCase CRM, which had the same R_f as seen with cells grown in the minimal media (Fig. 5). When the arginine auxotroph BR85 was grown in nutrient broth no such peak of CRM was seen (Fig. 5). This was expected, since this strain has been shown to lack OTCase activity (19). Extracts of BR85 showed some precipitated radioactive material near the dye front of the SDS-gels. This could also be seen in extracts of strain 168 from late culture times in supplemented nutrient broth (13 to 14 h and onwards; data not shown). This material was not truly cross-reactive with OTCase, because it was also bound nonspecifically to the immunoprecipitate formed by bovine serum albumin and anti-bovine serum albumin in cell extracts (data not shown). In addition, when increasing amounts of nonradioactive OTCase were added to extracts before immunoprecipitation, the amount of radioactivity at a position in the gels corresponding to M_r = 44,000 decreased progressively; the radioactivity at the dye front did not decrease (data not shown). Radioactivity at the dye front was greatly reduced in immunoprecipitates for which the cell extracts were centrifuged at 100,000 × g (4°C) for 1 h before immunoprecipitation, rather than at 20,000 × g (4°C) for 20 min as was done for the experiment illustrated in Fig. 5.

These data indicated that the same OTCase protein was produced in *B. subtilis* cells that were grown in the presence or absence of arginine and that, when OTCase activity was missing in strain BR85, OTCase protein was also missing. It was of interest to determine whether the high levels of OTCase activity in cells grown in the presence of arginine resulted from increased OTCase synthesis or from an activation of the same amount of enzyme as is formed when arginine was not present. *B. subtilis* strain 168 was grown in two minimal cultures (500 ml each) containing 0.1% (wt/vol) glucose. One culture contained all 19 amino acids at 50 μg/ml each and no arginine, and the other was grown in the same medium except that arginine was included at 150 μg/ml. L-[4,5-^{3}H]leucine was added to both cultures (4 μCi/ml) at the start of growth. Samples, 30 ml, were removed every 0.5 h between 3.5 and 6 h. The cells were harvested and washed, extracts were made, and OTCase was assayed. OTCase CRM was also determined by immunoprecipitation as described above. Measurement of the radioactivity remaining in the culture supernatant at the end of growth of each culture (6 h) showed that 53% of the added leucine still remained in the medium. Since the starting concentration of leucine was 50 μg/ml, this meant that sufficient leucine remained in the medium at the end of growth to repress the biosynthesis of nonradioactive leucine in the cells. The leucine incorporated into OTCase was thus of a constant specific radioac-

tivity throughout the course of growth. Under these conditions the radioactivity in the immunoprecipitates at a position in the gels corresponding to an M_r of 44,000 was a direct measure of amount of OTCase protein at the time the cells were harvested.

No OTCase CRM was present in the cells grown in the presence of arginine before enzyme activity appeared. The specific activity of the OTCase which then appeared was 87 ± 2 mU/cpm, as compared with a specific activity of 84 ± 4 mU/cpm for the OTCase from cells grown in the culture lacking arginine. The constant specific activity of the OTCase in cultures grown in the presence or absence of arginine demonstrated that the higher level of OTCase found in cultures containing arginine resulted from increased synthesis of OTCase, and not from activation of the enzyme found in the absence of arginine.

Is OTCase required for growth of *B. subtilis* cells on arginine? Figure 6 shows the growth of strain BR85 and its parent strain JH862 on arginine as the sole course of carbon and nitrogen. OTCase was not present at any time during the growth of BR85 in either well-aerated or poorly aerated cultures. In a well-aerated culture, OTCase activity could be found in strain JH862 only at the end of exponential growth (8 h) before the cells lysed. In a poorly aerated culture, strain JH862 showed OTCase activity only at 22 h. The data showed that OTCase activity was not required for growth of *B. subtilis* on arginine, since BR85 grew as well as its parent. In strain JH862, OTCase was induced by arginine only at the beginning of stationary phase. This was true even though no other source of carbon or nitrogen was present. In addition, the enzyme could not be induced during exponential growth on arginine by reduced aeration. This was in contrast to findings with *B. licheniformis*, in which OTCase activity is induced by poor aeration (5).

Is OTCase required for the deposition of arginine in spores? *B. subtilis*, *B. licheniformis*, and *B. megaterium* spores are known to contain large amounts of arginine (21, 29). If OTCase is required in *B. subtilis* for the biosynthesis of arginine during sporulation, it might be expected that free arginine would be deficient in the spores of a mutant lacking OTCase. The protein-bound and free amino acid content of spores was determined in strain BR85, which lacks OTCase, and in its parent strain JH862, which does not lack OTCase. The levels of protein-bound and free amino acids in spores from both strains JH862 and BR85 were similar to those found by Nelson and Kornberg (21). There were large and roughly equimolar amounts of free glutamate and arginine (about 120 μmol per g of dry spores) and very low levels of other free amino acids. There was no deficiency in the level of either bound or free arginine in the spores of strain BR85.

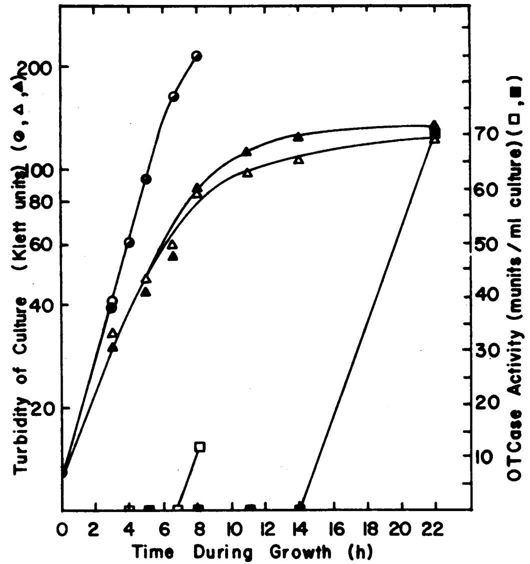

FIG. 6. Growth and OTCase activity of *B. subtilis* strains JH862 and BR85 growing on arginine. Strains JH862 and BR85 were grown on arginine as the sole source of carbon and nitrogen as described in the text. Growth of both organisms was coincident in the well-aerated cultures (◐). Symbols: (▲) growth of JH862 under poor aeration; (△) growth of BR85 under poor aeration; (□) OTCase activity in JH862 in the well aerated culture; (■) OTCase activity in JH862 in the poorly aerated culture. There was <0.1 mU of OTCase activity per ml of culture in BR85 in either the well-aerated or the poorly aerated cultures at any time.

DISCUSSION

Properties of *B. subtilis* OTCase. To our knowledge, this is the first report of purification of *B. subtilis* OTCase to near homogeneity. The preparations described by Issaly and Issaly (11) can be estimated on the basis of their specific activity to have been about 20% pure. The subunit molecular weight of the enzyme was 44,000 ± 3,000, a value close to that reported for OTCase from *B. licheniformis* (17). The native enzyme existed in multiple states of aggregation, whose molecular weights corresponded to dimeric, tetrameric, and hexameric states. It appears that these forms are associated in very stable form via disulfide bonds; in the presence of thiols the predominant form of the enzyme was the dimer. Issaly and Issaly (11) reported the molecular weight of *B. subtilis* OTCase to be 280,000, which is close to our value for the hexamer. Legrain et al. (17) found a value of

140,000 in studies with crude preparations. Issaly and Issaly (11) included 1 mM 2-mercaptoethanol in their buffers, but this may have been insufficient to reduce a relatively crude preparation. We found that pure OTCase adsorbed to agarose columns when 2-mercaptoethanol was present, so the dimeric form would be easily overlooked in gel filtration studies. We believe that failure to recognize the importance of thiols in determining the state of aggregation and the study of impure preparations accounts for the disagreements among the various workers.

Number of OTCases in *B. subtilis*. Although *B. subtilis* OTCase is both repressed by arginine during exponential growth and induced by arginine in the stationary phase, this organism does not produce two OTCases, i.e., a biosynthetic OTCase and a catabolic OTCase. The evidence for this conclusion is as follows. No OTCase activity or cross-reactive protein was ever detected in the arginine auxotrophic strain BR85, whether the cells were grown on limiting arginine, on excess arginine into stationary phase, or sporulating in nutrient broth. Immunochemical characterization of the OTCase produced in the parent strain under these conditions and in cells growing exponentially without arginine also showed that a single enzyme was formed. The CRM found always had a subunit molecular weight of 44,000 and reacted with antibodies directed against pure OTCase (which was purified from cells grown under inducing conditions). The specific activity (catalytic activity per microgram of cross-reactive protein) was the same in cells grown in the absence of arginine and under inducing conditions.

The alternative possibility, namely, that strain BR85 is a regulatory mutant preventing the synthesis of two very similar OTCases, is unlikely. Mahler et al. (19) have presented genetic evidence that the *argC* locus (of which strain BR85 is a representative) is the structural gene for OTCase and that no regulatory loci lie adjacent to it. Furthermore, if strain BR85 were a regulatory mutant, it would be expected to be affected in the activity of the arginine-repressible carbamyl phosphate synthetase, which maps adjacent to *argC* (24) and also shows "induction" by arginine in stationary cells (24). However, the arginine-repressible carbamyl phosphate synthetase shows normal activity and repression in strain BR85 (Paulus, Ph.D. thesis). The ability of arginine to both repress and stimulate synthesis of a single biosynthetic OTCase has been carefully studied with *Escherichia coli* B (13, 14). In this case, evidence was presented that arginine can act to both activate and inhibit the *argR* product, which is probably a repressor protein capable of complex interactions. We suggest that a similar mechanism may operate in *B. subtilis*.

The data for *B. subtilis* presented in this study call into question the conclusion that *B. licheniformis* has two different OTCases. The conditions used to induce biosynthetic and "catabolic" OTCase in *B. licheniformis* were essentially the same as those used to obtain "biosynthetic" and induced OTCase in the present study. Laishley and Bernlohr were unable to demonstrate any physical or kinetic differences between the two enzymes (16). The two OTCase activities fractionated slightly differently during ammonium sulfate precipitation, but these differences and differences in heat stability may have been due to the different composition of the crude extracts used as a source of each enzyme. Legrain et al. (17) purified catabolic OTCase from *B. licheniformis*. The enzyme was purified 140-fold from stationary-phase cells and was 95% pure as judged by SDS-polyacrylamide gel electrophoresis. The pure enzyme showed a subunit molecular weight of 44,000 and a native molecular weight of 140,000, as determined by molecular sieving. The type of gel filtration column used and the presence or absence of thiols were not stated. The anabolic OTCase from *B. licheniformis* was also reported to have a native molecular weight of 140,000 (17), but the method used to make this determination and the source or purity of the preparation were not stated. Thus, there is little direct evidence that the anabolic and catabolic OTCases of *B. licheniformis* are physically distinct entities. We believe that the possibility should be considered that this organism, like *B. subtilis*, produces a single OTCase whose activity can be both repressed and induced by arginine.

Physiological roles of OTCase in *B. subtilis*. One role for *B. subtilis* OTCase, namely, biosynthesis of arginine in cells growing without this amino acid, is firmly established (19). Does OTCase also play a role in arginine catabolism? The only evidence that it does so is that OTCase is induced by arginine at the end of exponential growth, when glucose is exhausted and aeration is less efficient in batch cultures. The arginine-repressible carbamyl phosphate synthetase of *B. subtilis*, which presumably can serve only a biosynthetic function, is regulated in the same manner, however (24). Thus, such regulation is not a compelling argument for a catabolic role, especially since *B. subtilis* is known to possess the arginase pathway for arginine catabolism.

Evidence against a catabolic role for OTCase in *B. subtilis* comes from reports that this organism lacks the arginine deiminase pathway (17). We showed in this report that wild-type cells and a mutant lacking OTCase grew equally well on arginine as the source of carbon and nitrogen. Furthermore, OTCase only appeared at the end

of exponential growth under these conditions in the wild-type strain. This result proved that OTCase synthesis was not repressed by glucose. Clearly, the arginase pathway was operative in *B. subtilis* cells growing on arginine. The deiminase pathway, if it exists at all, was dispensable and apparently repressed. It would be interesting to determine whether a *B. subtilis* mutant lacking arginase can grow on arginine under any conditions.

B. subtilis spores are known to store large amounts of free arginine (21). Since a degradative role could not be assigned to OTCase, we considered that it might be needed at the end of exponential growth to synthesize arginine for incorporation into spores. The levels of free and protein-bound amino acids in spores of *B. subtilis* strain JH862 were compared with those in strain BR85, which lacked OTCase and was therefore unable to synthesize arginine. No significant differences could be found. Strain BR85 sporulated as well as its parent, since it produced the same amount of spores in nutrient broth.

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