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Stabilization and Purification of Ornithine Transcarbamylase from *Neisseria gonorrhoeae*

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Ornithine transcarbamylase was stabilized in cell-free extracts by the presence of either carbamyl phosphate or glycerol. The enzyme was rapidly purified by a procedure consisting of ion-exchange chromatography and electrofocusing. The native molecular weight of the enzyme was determined by gel filtration to be 110,000. A subunit molecular weight of 36,000 was determined by polyacrylamide electrophoresis under dissociating conditions. These findings indicated a trimeric quaternary structure for the enzyme. The isoelectric point of the purified enzyme was 4.75, and no evidence of multiple forms of active enzyme was found in either crude or purified preparations. An inactive form of the enzyme appeared upon storage in the absence of stabilization buffer.

Nutritional auxotyping of clinical isolates of *Neisseria gonorrhoeae* has been well described by Carifo and Catlin (4), and this classification system has been useful in epidemiological studies (7, 10). A multiple auxotroph, requiring arginine, hypoxanthine, and uracil, is a frequently encountered auxotype and is intriguing because of the existence of a common metabolic link between the two biosynthetic pathways involved. Carbamyl phosphate is a branch-point metabolite shared by both the arginine and pyrimidine pathways and enters the arginine pathway by the following reaction: ornithine + carbamyl phosphate → citrulline + P_i. The reaction is catalyzed by ornithine transcarbamylase (carbamyl phosphate:L-ornithine carbamyl transferase, EC 2.1.3.3). The enzyme has been well studied in a variety of microorganisms (1, 6, 8, 9, 11-13, 17-19, 26), but a study of the enzyme from *N. gonorrhoeae* has not been reported. Catlin and co-workers (6, 25) studied arginine biosynthesis in over 200 clinical isolates of *N. gonorrhoeae*, but the enzymes of the pathway were not characterized.

The present study was undertaken to establish the basis for further defining the interlocking relationships between the arginine and pyrimidine biosynthetic pathways. Conditions for stabilizing the enzyme were determined, and the enzyme was subsequently purified and partially characterized.

MATERIALS AND METHODS

Bacterial strain. The standard laboratory strain of *N. gonorrhoeae*, F-62 (colony type 4), was obtained from Edward R. Gubish, Department of Microbiology and Immunology, Baylor College of Medicine. This strain does not require either arginine or pyrimidines for growth.

Preparation of enzyme extracts. The organism was maintained on gonococcal base agar plus 1% IsoVitaleX in a candle jar at 37°C, and stock plates were passaged daily. Cultures for cell-free extracts were grown in Catlin's defined medium (5) minus L-arginine, hypoxanthine, and uracil. Cells from a 5-liter culture were harvested in late exponential phase of growth by centrifugation, washed once with 50 ml of 50 mM triethanolamine-hydrochloride buffer (pH 8.0), and stored at −70°C. The cell pellet was suspended in 10 ml of 50 mM triethanolamine buffer (pH 8.0) plus 20% (vol/vol) glycerol (stabilization buffer). Disruption of the cells was achieved by passage through a French pressure cell (Aminco, Silver Spring, Md.) at 4°C. The cellular debris was removed by centrifugation at 35,000 × *g* for 30 min. The supernatant solution was the cell-free extract used in the subsequent purification procedure.

Ornithine transcarbamylase assay. Activity was measured by a modification of the method of Prescott and Jones (23). The reaction mixture contained 0.5 μmol of carbamyl phosphate (dilithium salt), 0.5 μmol of L-ornithine, 10 μmol of triethanolamine buffer (pH 8.0), 20% (vol/vol) glycerol, and an appropriate amount of enzyme in a final volume of 0.2 ml. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 0.8 ml of chromogenic reagent. The reaction tubes were heated at 95°C for 15 min, after which they were cooled to room temperature. The absorbance at 466 nm was determined by a Gilford spectrophotometer. Two reaction tubes, one minus ornithine and one minus enzyme, were included to correct for any background color. The chromogenic reagent consisted of two solutions, A and B, mixed in equal proportions before use. Solution A consisted of 0.85 g of antipyrine dissolved in 100 ml of 40% (vol/vol) H_2SO_4. Solution B contained 0.62 g of 2,3-butanedione monoxime dissolved in 100 ml of 5% (vol/vol) acetic acid. One unit of activity was defined as the amount of ornithine transcarbamylase that produces 1 nmol of citrulline per min at 37°C under standard laboratory conditions. Specific activity was defined as units per milligram of protein. Protein concentration

was determined by the method of Lowry et al. (15), using bovine serum albumin as the standard.

Purification of ornithine transcarbamylase. Step 1. Ion-exchange chromatography. Ten milliliters of cell extract was applied to a column of DEAE-cellulose (2.5 by 25 cm) equilibrated at 25°C in 10 mM triethanolamine buffer (pH 8.0) containing 20% (vol/vol) glycerol (dilute stabilization buffer) according to Whatman Technical Bulletin 1E2. The column was washed with 100 ml of the dilute stabilization buffer to remove unadsorbed material. Selective elution of the adsorbed proteins was achieved by using a 300-ml linear gradient of KCl (0.1 to 0.3 M) in dilute stabilization buffer, and 1-ml fractions were collected. The enzyme eluted at a KCl concentration of 0.2 M. The fractions containing 80% of the activity were pooled, concentrated, and dialyzed against stabilization buffer in an Amicon 52 Ultrafiltration cell (PM 10 membrane).

Step 2. Electrofocusing. A linear glycerol gradient containing 2% LKB ampholine (pH 4 to 6.5) was prepared in an LKB (model 8101) electrofocusing column. Ornithine transcarbamylase from step 1 was mixed into the dense solution before introduction into the column. The run was conducted at 4°C with a constant power setting of 10 W for 15 h. When focusing was complete, 1-ml fractions were collected, and the pH of each fraction was determined at 4°C. The enzyme-containing fractions were dialyzed against the stabilization buffer and concentrated by ultrafiltration as described in step 1. This purified preparation was utilized in subsequent characterization studies.

Native molecular weight. The native molecular weight of the enzyme was determined by gel filtration. One milliliter of the enzyme from step 2 was applied to an AcA 34 column (1.5 by 60 cm) which had been equilibrated in stabilization buffer plus 100 mM KCl. The enzyme was chromatographed (descending) at 25°C at a flow rate of 12 ml/h, and 1-ml fractions were collected. The column was calibrated for molecular weight determinations according to Andrews (2), using the following standards: catalase (molecular weight, 232,000), immunoglobulin G (150,000), human ornithine transcarbamylase (110,000), alkaline phosphatase (80,000), and bovine serum albumin (67,000).

Electrophoresis. Native ornithine transcarbamylase was subjected to discontinuous electrophoresis according to the procedure of Maizel (16). Polyacrylamide (7.5%) gels were prepared with a Tris-hydrochloride buffer (pH 8.9), and the upper and lower electrode solutions consisted of Tris-glycine (pH 8.3). The gels were run at a constant current of 3 mA per gel at 4°C for 2 h. The gels were fixed, stained with Coomassie blue, and destained in the standard manner.

Electrophoresis under dissociating conditions was run on Bio-Rad precast gels according to the manufacturer's recommendations (Bulletin 1038). The molecular weight of the ornithine trnascarbamylase polypeptide was determined by the method of Weber and Osborn (27). The molecular weight standards were: phosphorylase b (molecular weight, 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,000).

Electrofocusing in polyacrylamide. Electrofocusing of the enzyme in 5% polyacrylamide cylindrical gels was performed by the method of Wrigley (28) using riboflavin (0.05%) to effect polymerization. The gels contained 2% ampholine (pH 3 to 10) in a final concentration of 10% glycerol. One hundred units of enzyme activity (50% glycerol) was layered on the gels and overlaid with 25 μl of 10% glycerol. The upper and lower reservoirs contained 0.02 M NaOH and 0.01 M H_3PO_4, respectively, and electrofocusing was carried out by stepwise increases in voltage (100 V for 30 min, 300 V for 2 h, and then 500 V for 30 min).

Detection of ornithine transcarbamylase in polyacrylamide gels. Enzyme activity was determined in indicated experiments by removing the gels after electrophoresis or electrofocusing and incubating for 15 min in a substrate mixture (2.5 mM ornithine and 2.5 mM carbamyl phosphate). The inorganic phosphate produced by the enzyme reaction was visualized by the lead-sulfide procedure described by Baron and Buttery (3).

L-Ornithine, Triton X-100, triethanolamine, amino acids, and molecular weight standards for gel chromatography were obtained from Sigma Chemical Co. Chemicals and molecular weight standards for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories. DEAE-cellulose was obtained from Whatman. Gonococcal base agar and IsoVitaleX was from BBL Microbiology Systems. Electrofocusing materials and gel chromatography material (AcA 34) were purchased from LKB. All other chemicals were of the highest purity commercially available.

RESULTS

Stabilization of enzyme activity. Initial attempts to purify ornithine transcarbamylase from *N. gonorrhoeae* were hampered by the enzyme's instability. However, enzyme stability was achieved by two different means (Fig. 1). The addition of 20% (vol/vol) glycerol to the triethanolamine buffer (pH 8.0) stabilized the enzyme in crude cell-free extracts for at least 2 weeks. Alternatively, carbamyl phosphate, a substrate, also stabilized the enzyme for a similar time period. The other substrate, ornithine, was a much less effective stabilizing agent, and 50% of the activity was lost within 24 h in the absence of any additions (Fig. 1). Enzyme concentration also played a role in stabilization, and protein concentrations less than 0.2 mg/ml resulted in rapid reduction of enzyme activity even in the presence of glycerol or carbamyl phosphate. Routinely, 20% (vol/vol) glycerol was used as the stabilizing agent in a triethanolamine buffer (pH 8.0), and the protein concentration was maintained above 0.5 mg/ml. Triethanolamine was used as the buffer because Tris, which also buffers well at pH 8.0, inhibited the enzyme; 50% inhibition of enzyme activity was observed in 50 mM Tris. Inhibition of ornithine transcarbamylase from human liver by Tris has been reported (22).

Purification of ornithine transcarbamylase. The first step in the purification of the enzyme consisted of DEAE chromatography. The enzyme eluted off as a single peak at a KCl concentration of 0.2 M. Subsequently, the enzyme was subjected to electrofocusing in a preparative glass column over a narrow pH gradient (pH 4 to 6.5). The results of several purification experiments are summarized in Table 1. The purification process required only 26 h.

Electrofocusing. Preparative electrofocusing served two important functions. First, it was effective as a purification step (Table 1), and second, it allowed for the determination of the isoelectric point (pI) of the enzyme. The enzyme focused into one peak of activity with a pI of 4.75 (Fig. 2). No evidence of multiple forms of the enzyme was found, even when a crude cell-free extract was electrofocused over a pH 3 to 10 gradient (data not shown).

Molecular weight and subunit composition. The native molecular weight of the purified enzyme was determined by gel filtration to be 110,000. No molecular weight changes in the enzyme were observed when the organism was grown in the presence of L-arginine (5 mg/ml).

A subunit molecular weight of 36,500 was determined by electrophoresis under dissociating conditions as described in Materials and Methods. This finding, together with the data from the native molecular weight studies, indicates that the enzyme exists as a trimer of three similar-size subunits. This is consistent with several previous studies of ornithine transcarbamylase from bacterial and mammalian sources (11, 12, 21, 22, 26).

Evaluation of purified ornithine transcarbamylase. The purified enzyme was analyzed by analytical electrofocusing in cylindrical polyacrylamide gels (Fig. 3). Active enzyme was detected by the activity stain described in Materials and Methods. The active enzyme migrated with the same relative mobility in both the unfractionated cell-free extract (data not shown) and the purified enzyme preparations. In addition, only one active form of the enzyme was observed in both preparations.

Formation of inactive form of the enzyme. Figure 4 indicates that the purified enzyme may undergo inactivation during storage. An electrophoretically distinct band appears in

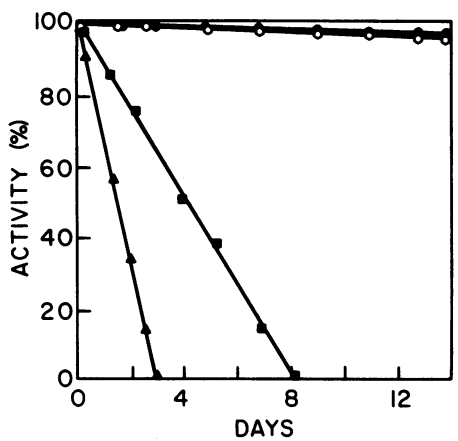

FIG. 1. *Stability studies. A cell-free extract was prepared in 50 mM triethanolamine buffer (minus glycerol) (pH 8.0), as described in the text. An appropriate amount of extract was dispensed into tubes containing the indicated additives in a total volume of 0.5 ml:* (▲) *50 mM triethanolamine;* (■) *50 mM triethanolamine plus 5 mM* L*-ornithine;* (●) *50 mM triethanolamine plus 20% (vol/vol) glycerol;* (○) *50 mM triethanolamine plus 1 mM carbamyl phosphate. The tubes were maintained at 4°C, and, at the indicated times, 0.02 ml was withdrawn and assayed for enzyme activity.*

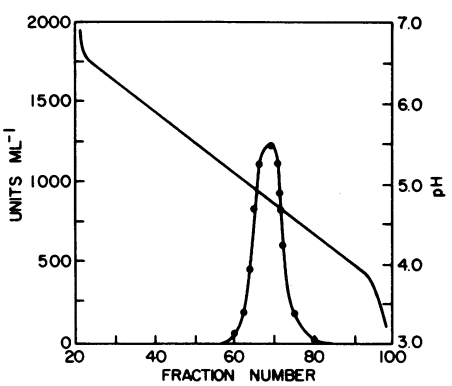

FIG. 2. *Electrofocusing (pH 4 to 6.5) of ornithine transcarbamylase. Enzyme from the DEAE step of the purification procedure was subjected to electrofocusing as described in the test.*

TABLE 1. *Purification of ornithine transcarbamylase*

Step	Total vol (ml)	Total units (nmol/min)	Total protein (mg)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	0.4	64,000	300.0	210	100	1
DEAE chromatography	80.0	45,000	36.0	1,200	70	6
Isoelectric focusing (pH 4 to 6.5)	14.0	21,000	1.2	16,400	32	77

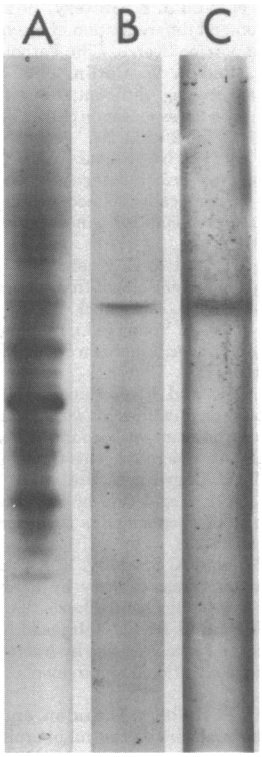

FIG. 3. *Electrofocusing of crude and purified ornithine transcarbamylase in polyacrylamide gels. Details are given in the text. (A) Protein stain of cell-free extract (25 μg); (B) protein stain of purified ornithine transcarbamylase (25 μg); (C) activity stain of ornithine transcarbamylase (100 U) preparation used in (B).*

the purified enzyme preparation upon storage in the absence of stabilization buffer. The intensity of the more rapidly migrating, inactive protein band seems to increase with storage time (data not shown). This is supported by an accompanying decrease in specific activity. Storage in stabilization buffer prevents this apparent conversion.

DISCUSSION

The present study established that ornithine transcarbamylase from *N. gonorrhoeae* can be stabilized by either of two methods: inclusion of 20% glycerol or inclusion of 1 mM carbamyl phosphate. Other commonly used enzyme-stabilizing compounds, such as dithiothreitol, inhibitors (e.g., P_i), EDTA, and protease inhibitors did not stabilize the enzyme. A minimal enzyme concentration (0.2 mg/ml) was also obligatory for stability. The same conditions also apply in stability of the purified enzyme. When the purified enzyme was stored in the absence of the stabilization buffer a more electrophoretically mobile inactive form of the enzyme appeared.

The specific activity for the gonococcal enzyme in unfractionated cell-free extracts was low relative to reported values of most other bacteria. However, the specific activity for the various bacterial ornithine transcarbamylases ranges from 256 nmol/min per mg of protein in *Bacillus subtilis* (20) to 256,000 nmol/min per mg in *Streptococcus faecalis* (17).

The enzyme exhibited a native molecular weight of 110,000 and a subunit molecular weight of 36,500. Thus, we propose that ornithine transcarbamylase from *N. gonorrhoeae* exists as a trimer of similar-size subunits. This is consistent with the molecular weight and trimeric structure described for the anabolic enzymes of *Salmonella typhimurium* (1), *Escherichia coli* (12, 13), *Saccharomyces cerevisiae*

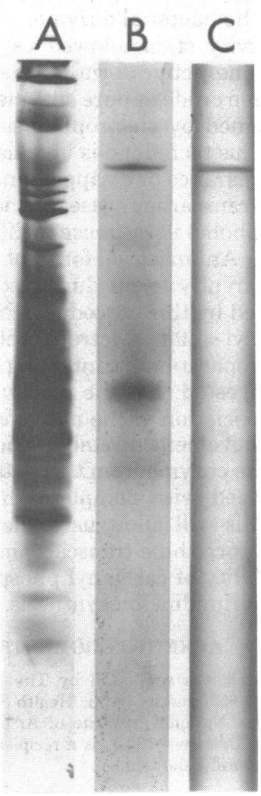

FIG. 4. *Polyacrylamide gel electrophoresis of crude and purified ornithine transcarbamylase. Two preparations were subjected to electrophoresis. (A) Protein stain of cell-free extract (25 μg); (B) protein stain of purified enzyme (25 μg) (stored in absence of stabilization buffer); (C) activity stain (see the text) of ornithine transcarbamylase (100 U) preparation used in (B).*

(21), and *Pseudomonas fluorescens* (26). These findings are in good agreement with the enzyme from human liver, which suggests that an evolutionary advantage exists for the conservation of the unusual quaternary structure. Interestingly, the catabolic enzymes of *P. fluorescens* (8), *S. faecalis* (17), and *Mycoplasma hominis* (24) have molecular weights of 312,000, 228,000, and 360,000, respectively, and the quaternary structure of the enzymes from the first two sources consists of six identical subunits. The exception to this functional relationship seems to be the apparent anabolic enzyme of *B. subtilis*, which has a molecular weight of 280,000 (9). However, this value could not be reproduced by Legrain et al. (14), who obtained a molecular weight of 140,000 for the enzyme from *B. subtilis*.

The current study also established the isoelectric point (4.75) of ornithine transcarbamylase, which, to our knowledge, is the first report of the pI for the bacterial enzyme.

The activity stain allowed us to determine that only one active form of the enzyme was evident when crude or pure enzyme preparations were examined by electrophoresis and electrofocusing. This technique is invaluable in assessing the appearance or disappearance of different ornithine transcarbamylase species (e.g., catabolic or anabolic) in response to different growth conditions. An inactive form of the enzyme, which apparently forms during storage at 4°C, was detected by this procedure. Preliminary sodium dodecyl sulfate electrophoretic analysis on enzyme preparations containing the inactive species suggested that the inactive form represents a dissociation of the native enzyme.

The achievement of stabilization and purification of the enzyme from this pathogen enables us to proceed with complete characterization studies. This will allow us to determine what role, if any, ornithine transcarbamylase plays in the availability of carbamyl phosphate for arginine and pyrimidine biosynthesis.

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