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Purification and Characterization of Orotidine-5'-Phosphate Decarboxylase from *Escherichia coli* K-12

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Using blue Sepharose affinity chromatography, we purified orotidine-5'-phosphate decarboxylase over 600-fold, to near homogeneity, from strains of *Escherichia coli* harboring the cloned *pyrF* gene on the multicopy plasmid pDK26. The purified enzyme has a subunit molecular weight of 27,000 but appears to be catalytically active as a dimer. In contrast to yeast enzymes, orotidine-5'-phosphate decarboxylase from *E. coli* is unstable at pH 6.0. The specific activity and K_m values were 220 U/mg and 6 μM, respectively.

Orotidine-5'-phosphate decarboxylase (OMPase) (EC 4.1.1.23) catalyzes the conversion of orotidine-5'-phosphate (OMP) to uridine monophosphate in the de novo biosynthesis of pyrimidines (9). Umeza et al. (15) reported a 6,000-fold purification of the enzyme from bakers' yeast by conventional techniques. The molecular weight of the native enzyme, as determined by Sephadex G-100 gel filtration, was reported as 51,000.

Handschumacher (4) has reported that an analog of OMP, 6-azauridine 5'-phosphate, is a competitive inhibitor of OMPase. Accordingly, Brody and Westheimer (3) used a derivative of azauridine to synthesize an affinity resin for OMPase. The enzyme bound tightly and was specifically eluted with azauridine, effecting a 3,200-fold purification from bakers' yeast homogenate in one pass. Their purified enzyme had a subunit molecular weight of 27,000 and specific activity and K_m values of 36 U/mg and 0.5 μM, respectively.

Certain enzymes have been shown to bind tightly to Cibacron Blue F3GA conjugated to dextran (blue dextran) (1, 6, 13, 14). It has been proposed that these enzymes complex with blue dextran because of a structural similarity between Cibacron Blue F3GA and nucleotides (5, 10, 12). Using this method, Reyes and Sandquist (11) purified OMPase from crude yeast extracts ca. 6,000-fold with either OMP or uridine monophosphate as the eluting ligand.

Recently, the *E. coli* structural gene for OMPase (*pyrF*) has been cloned and physically characterized (W. P. Donovan and S. R. Kushner, Gene, in press). When grown in the absence of pyrimidines, *pyrF* strains of *E. coli* which harbored the cloned gene had ca. 60-fold higher levels of enzyme activity than $pyrF^+$ control strains without the plasmid. This study reports the first purification and characterization of OMPase from *E. coli*. The procedure combines the advantages of gene amplification with use of blue Sepharose affinity chromatography. Purified OMPase from *E. coli* was found to have a subunit molecular weight similar to that reported for the yeast enzyme. However, the specific activities of the two enzymes as well as their stabilities at pH 6.0 differed significantly.

MATERIALS AND METHODS

Assay for OMPase. The enzyme assay is based on the decrease in absorption at 285 nm which occurs when OMP is converted to uridine monophosphate by OMPase ($\Delta\epsilon = 2.25 \times 10^3\ M^{-1}\ cm^{-1}$) (3, 9, 15). The standard assay mixture (1 ml) contained 75 μM OMP plus ca. 0.01 U of the enzyme in 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol. The rate of decrease in absorbance was measured with a Beckman Acta recording spectrophotometer. One unit of enzyme activity was defined as the conversion of 1 μmol of OMP to uridine monophosphate in 1 min. Enzyme concentrations were determined either by the method of Warburg and Christian (16) and Layne (8) or by comparison with standards after silver staining of polyacrylamide gels.

Kinetics. The V_{max} and K_m values were determined by assay in 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol containing 2 to 75 μM substrate and ca. 0.01 U of the purified enzyme. For pH optimum and pH stability the assay mix contained 5 mM β-mercaptoethanol, 75 μM substrate, ca. 0.01 U of purified enzyme, and 100 mM of either 2(*N*-morpholino)ethanesulfonic acid (pH 5.5 to 6.0), morpholinopropanesulfonic acid (pH 6.0 to 7.0), potassium phosphate (pH 6.0), or Tris-hydrochloride (pH 7.5 to 9.0) buffer. The pH optimum for enzyme activity was determined by diluting a sample of the purified enzyme (stored in 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol) 100-fold in the appropriate pH buffer (100 mM) containing 5 mM β-mercaptoethanol and 75 μM OMP and assaying immediately. Enzyme stability at various pHs was determined in a similar manner except that OMP was not added and the activity was

not assayed until 30 min after the purified enzyme had been diluted in the various pH buffers.

Materials. 6-Azauridine 5'-phosphate and OMP were obtained from Calbiochem. The blue Sepharose affinity column was prepared by attaching Cibacron Blue F3GA dye (Sigma Chemical Co.) to Sepharose by the method of Bohme et al. (2). Silver nitrate was purchased from Accurate Chemical Co.

Purification of OMPase. All of the steps, except where indicated, were carried out at 0 to 4°C. The procedures for ammonium sulfate precipitation and for elution of OMPase with azauridine were modeled after those of Brody and Westheimer (3).

(i) **Cell lysis.** Strain SK4766 (*pyrF*::Tn*5* Kan^r/pDK26 *bla*$^+$*pyrF*$^+$) (Donovan and Kushner, in press) was grown in 8 liters of Luria broth (growth in rich medium did not change OMPase levels, data not shown) plus ampicillin to a Klett reading of ca. 200 (no. 42 green filter, 5 × 10^8 cells per ml). After centrifugation, 27 g of cells were suspended in 108 ml of 50 mM Tris-hydrochloride (pH 7.8)–10% sucrose (wt/vol). Lysozyme (108 mg) plus 27 ml of 200 mM EDTA, pH 8.0, were added and cells were incubated at room temperature for 15 min. The lysate was then centrifuged in a Beckman 50 Ti rotor at 80,000 × *g* for 45 min and the supernatant (fraction I) was collected.

(ii) **Ammonium sulfate fractionation.** Fraction I was brought to 45% saturation with ammonium sulfate and centrifuged in a Beckman JA20 rotor at 12,100 × *g* for 15 min. The supernatant was brought to 77% saturation with ammonium sulfate and centrifuged as before, and the precipitate was suspended in 20 ml of 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol. The solution was dialyzed against 64 mM Tris (pH 7.8)–5 mM β-mercaptoethanol for 6 h (fraction II).

(iii) **Affinity chromatography.** Fraction II was centrifuged at 12,100 × *g* for 15 min to remove a white precipitate which formed during dialysis. The supernatant (38 ml) was then applied to a 1.9- by 24.5-cm blue Sepharose column (69-ml bed volume). OMPase was eluted with 700 ml of a 20 to 80 mM NaCl gradient in 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol. Starting with the application of the salt gradient, 3-ml fractions were collected and those containing greater than 0.3 U of activity (62 to 101) per ml were pooled and dialyzed against 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol (fraction III).

The blue Sepharose column was regenerated, and fraction III (114 ml) was reapplied to the column. The column was then washed with ca. 600 ml of 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol, and OMPase was subsequently eluted with 30 ml of the same buffer containing 100 μM azauridine. Fractions (1 ml each) were collected starting with the addition of the enzyme preparation, and fractions containing activity were pooled (fraction IV).

(iv) **Sephadex G-100 chromatography.** A small portion (0.5 ml) of fraction IV was applied to a Sephadex G-100 column (1.6 by 24 cm) equilibrated with either 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol or 100 mM KPO_4 (pH 6.0)–5 mM β-mercaptoethanol, and the enzyme was eluted with the same buffer (fraction V).

(v) **Polyacrylamide gel electrophoresis and protein staining.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5% gels was performed by the method of Weber et al. (17), using the Tris-hydrochloride and the Tris-glycine buffers described by Laemmli and Favre (7). The silver staining method of Wray et al. (18) was used to visualize proteins in acrylamide gels.

RESULTS

Elution of OMPase from blue Sepharose. When a 20 to 80 mM salt gradient in 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol was passed through the blue Sepharose column, OMPase was eluted in a broad peak at ca. 40 mM NaCl. Fractions containing greater than 0.3 U of activity per ml were pooled, dialyzed to remove NaCl, and reapplied to the blue Sepharose column. Over 90% of the applied enzyme activity was eluted in a narrow peak when a solution of Tris buffer containing 100 μM azauridine was passed through the column. A summary of the purification is provided in Table 1.

Gel electrophoresis. When fraction II was electrophoresed through a polyacrylamide gel and stained with silver, a minor band of 27,000 daltons was detected (Fig. 1, lane A). After salt elution of the enzyme from blue Sepharose, OMPase was one of the major proteins in the enzyme preparation (Fig. 1, lane B). Azauridine elution of OMPase from the blue Sepharose column gave an enzyme preparation that was estimated by visual examination of various stained gels to be greater than 98% pure (Fig. 1, lane C). A slight amount of contaminating protein was still visible after the enzyme was eluted from a Sephadex G-100 column (Fig. 3, lane D). On sodium dodecyl sulfate-polyacrylamide gels, purified OMPase had a molecular weight of 27,000.

V_{max} and K_m. When assayed in 64 mM Tris (pH 7.8)–5 mM β-mercaptoethanol, the purified enzyme had a specific activity of 220 U/mg and a K_m for OMP of 6 μM.

pH optimum and stability. Within experimental error, no distinct pH optimum was observed for OMPase when the enzyme was assayed from pH 5.5 to 9.0. However, when the enzyme was incubated at each pH at room temperature for 30 min before assaying as described above, significantly lower activities were found at pH 5.5 and 6.0.

Stability of OMPase at pH 6.0 and 7.8. Samples of fraction V, which had been stored at 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol, were diluted 16-fold in either 64 mM Tris (pH 7.8)–5 mM β-mercaptoethanol or in 100 mM potassium phosphate (pH 6.0)–5 mM β-mercaptoethanol. The diluted samples were incubated at 26°C and, at various time intervals, were assayed under standard conditions (see above). When preincubated at pH 7.8, the enzyme was slightly unstable at 26°C, but after 90

TABLE 1. Purification of orotidine-5'-phosphate decarboxylase

Fraction	Purification step	Vol (ml)	Total activity (U)	Total[a] protein (mg)	Sp act (U/mg)	Recovery (%)	Purification factor
I	Cell lysis	150	373	1,035	0.36	100	
II	Ammonium sulfate	38	295	274	1.08	79	3.0
III	Blue Sepharose-salt elution	114	149	23	6.48	40	18
IV	Blue Sepharose-azauridine	15	142	0.6[b]	220	38	611
V	Sephadex G-100	6	109	0.5	220	29	611

[a] From comparison with standards after silver staining of polyacrylamide gels and from the ratio of absorbance at 260 nm to absorbance at 280 nm by the method of Warburg and Christian (16) and Layne (8).

[b] Protein determination was made after passing a sample over a Sephadex G-25 column to remove azauridine.

min, ca. 80% of the original activity still remained (Fig. 2). Incubation at pH 6.0 quickly inactivated the enzyme such that after 90 min <1% of the original activity remained (Fig. 2). When 100 μM azauridine was included in the incubation mix, the enzyme was significantly stabilized at pH 6.0 with ca. 65% of the original activity remaining after 90 min (Fig. 2). It should be noted that fraction V lost little activity when stored in pH 7.8 buffer for several weeks at 0°C.

Elution of OMPase from a Sephadex G-100 column. When a 0.5-ml sample of fraction IV was applied to a Sephadex G-100 column (1.6 by 24 cm) and eluted with 64 mM Tris-hydrochloride (pH 7.8)–5 mM β-mercaptoethanol, the enzyme activity peak corresponded to a molecular weight of ca. 54,000 (Fig. 3). In contrast, if the enzyme was eluted with 100 mM potassium phosphate (pH 6.0)–5 mM β-mercaptoethanol, the elution profile suggested a molecular weight of ca. 27,000 (Fig. 3). A broad peak of activity was seen at an apparent molecular weight which was intermediate between 54,000 and 27,000 (Fig. 3) when the enzyme was eluted with 100 mM potassium phosphate (pH 6.0)–5 mM β-mercaptoethanol containing 100 μM azauridine. Approximately, 75, 16, and 60% of the applied activity was recovered when the enzyme was eluted with either 64 mM Tris (pH 7.8), 100 mM potassium phosphate (pH 6.0), or 100 mM potassium phosphate (pH 6.0) containing 100 mM azauridine, respectively.

DISCUSSION

OMPase has been purified over 600-fold from a strain of *E. coli* (SK4766) harboring the cloned *pyrF* gene. When 5 μg of the purified enzyme was stained with silver after electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel, a single major band, corresponding to a molecular weight of 27,000, was seen together with a barely detectable impurity (Fig. 1, lane

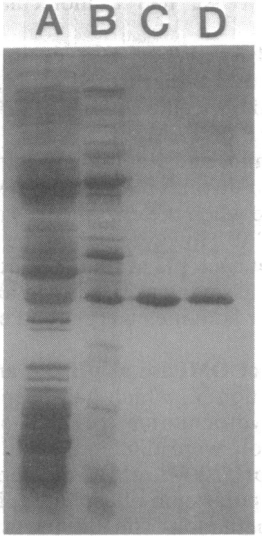

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel of various OMPase fractions. The gel was stained by the method of Wray et al. (18). Lanes: A, fraction II; B, fraction III; C, fraction IV; D, fraction V.

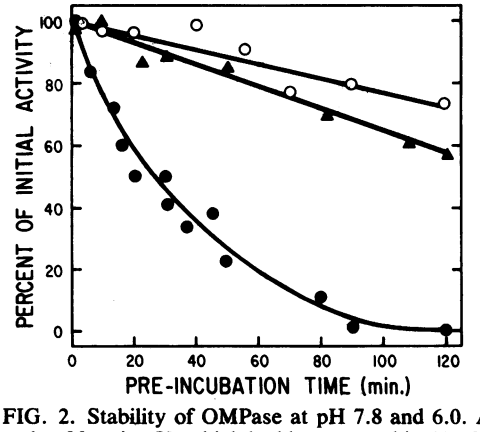

FIG. 2. Stability of OMPase at pH 7.8 and 6.0. A sample of fraction V, which had been stored in 64 mM Tris (pH 7.8)–5 mM β-mercaptoethanol, was diluted 16-fold in either 64 mM Tris, pH 7.8 (○), 100 mM potassium phosphate, pH 6.0 (●), or 100 mM potassium phosphate containing 100 mM azauridine, pH 6.0 (▲) (all buffers contained 5 mM β-mercaptoethanol) and subsequently was incubated for the indicated times at 26°C before being assayed under standard conditions.

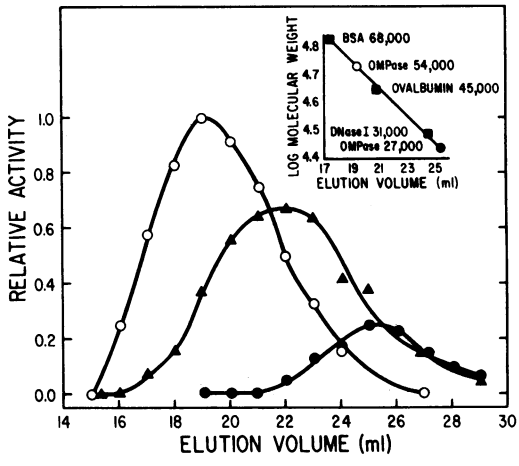

FIG. 3. Elution profiles of OMPase from Sephadex G-100 at either pH 7.8 or 6.0. An equal amount of enzyme (fraction IV) was applied to a Sephadex G-100 column (1.6 by 24 cm) equilibrated with either 64 mM Tris, pH 7.8 (○), 100 mM potassium phosphate, pH 6.0 (●), or 100 mM potassium phosphate containing 100 mM azauridine, pH 6.0 (▲) (all buffers contained 5 mM β-mercaptoethanol). Samples were eluted with the same buffer that was used to equilibrate the column. Fractions (1 ml each) were collected and assayed for OMPase under standard conditions. Relative activity was calculated by dividing the activity of each fraction (units per milliliter) by the peak activity (units per milliliter) obtained when the enzyme was eluted with 64 mM Tris, pH 7.8.

D). From additional gels, it is estimated that the protein preparation is greater than 98% pure (data not shown). Previous maxicell analysis of strains which harbored the cloned *pyrF* gene showed a major band corresponding to a molecular weight of 27,000 (Donovan and Kushner, in press). Since *pyrF* is contained on a 1,200-base pair DNA fragment (Donovan and Kushner, in press), it is most probable that OMPase consists of two identical subunits of molecular weight 27,000.

The enzyme was found to have a broad pH optimum. When assayed immediately after the addition of enzyme to the reaction mixture, no significant difference in activity was found from pH 5.5 to 9.0. However, when OMPase was preincubated for 30 min at each pH before assaying, the activities at pH 5.5 and 6.0 were less than 20% of the optimal value. In contrast, OMPase from yeast cells was reported to be quite stable at these pHs (3, 15).

The specific activity was 220 U/mg for the purified enzyme. This contrasts significantly with the specific activity of 36 U/mg reported for OMPase purified from yeast cells (3).

The native molecular weight determinations of OMPase at various pHs provide some insight into the subunit composition of the catalytically active enzyme. When the enzyme was eluted from a sizing column at pH 7.8, the peak of activity corresponded to a molecular weight of 54,000 (Fig. 3). However, elution at pH 6.0 yielded a single reduced peak of activity at the monomeric molecular weight of 27,000. The data presented in Fig. 2 suggest that in the absence of substrate, the monomer is very unstable.

To help determine whether monomer was catalytically active, another G-100 column was run at pH 6.0 in the presence of azauridine (a substrate analog). Under these conditions the enzyme appears to exist in equilibrium between the monomeric and dimeric forms (Fig. 3). Thus, although it cannot be ruled out that the monomer can function catalytically, the activity observed when the enzyme was eluted at pH 6.0 most likely arose from reassociation to the dimeric form in the presence of substrate. Significantly, the data in Fig. 3 suggest that azauridine specifically stabilizes OMPase by holding the enzyme in the dimeric form.

Since the enzymes from yeast and *E. coli* differ significantly in their stabilities at pH 6.0 and in their specific activities, it seems likely that the amino acid sequences of the two enzymes differ significantly. This conclusion is strengthened by the observation that yeast and *E. coli* DNA coding sequences had less than 70% homology, as determined by cross hybridization (Donovan and Kushner, in press). This result, however, does not rule out amino acid conservation at the active site.

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