Activity of Yeast Orotidine-5'-phosphate Decarboxylase in the Absence of Metals*

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Yeast orotidine-5'-phosphate decarboxylase was recently shown to contain zinc and to be inhibited by zinc-complexing agents. When the gene for the yeast enzyme was expressed in Escherichia coli, the gene product was devoid of metal atoms but exhibited a specific activity and molecular mass similar to those of the enzyme obtained directly from yeast. This invalidates the hypothesis that zinc is involved in substrate decarboxylation. The zinc-free enzyme undergoes thermal inactivation at a somewhat lower temperature than does the zinc-containing enzyme isolated from yeast.

Orotidine 5'-phosphate (OMP) undergoes spontaneous decarboxylation in neutral aqueous solution with a half-time of approximately 78 million years at 25 °C (1). At the active site of OMP decarboxylase (ODCase, EC 4.1.1.23), this reaction proceeds with a half-time of 18 ms, producing UMP, the biosynthetic precursor of pyrimidine nucleotides (Fig. 1) (2). Because of the biological importance of this reaction, the unusual magnitude of the rate enhancement (1017-fold), and the absence of cofactors such as those used in the enzymatic decarboxylation of α-amino and 2-keto acids, the mechanism of action of this enzyme has attracted unusual interest.

Recently, yeast ODCase was found to contain zinc and to be inactivated by several zinc-complexing agents (3). Earlier, ODCase had been shown to be susceptible to very strong reversible inhibition by 6-CSNH2-UMP (6-thiocarboxamidouridine 5'-phosphate), shown in Fig. 1, but not by 6-CONH2-UMP (6-carboxamidouridine 5'-phosphate), consistent with the possible involvement of a metal ion in enzyme-substrate interaction (4). To investigate the possible role of zinc in the action of ODCase, we used a bacterial system to express the ura3 gene that encodes yeast ODCase. We find this gene product to be fully active but devoid of metal atoms, invalidating the hypothesis that zinc is involved in catalysis.

EXPERIMENTAL PROCEDURES

Enzyme Expression and Purification—ODCase was expressed in yeast using strain BJ5424 containing plasmid pG2U, which contains the ura3 gene under control of a galactose-inducible promoter (2). Purification of the enzyme made by the yeast expression system was performed as described earlier (2), except for the addition of an anion exchange chromatography step to increase the purity of the product. For expression of ODCase in Escherichia coli, the Saccharomyces cerevisiae ura3 gene of plasmid pH596 (8) was subcloned into the EcoRI site of Stratagene vector pBCSK+ following polymerase chain reaction amplification. This procedure yielded a ura3 gene with a unique NdeI site at the 5'-end and a BamHI site 3' to the ura3 termination codon that are compatible with unique expression vector endonuclease cleavage sites. The nucleotide sequence of the amplified ura3 gene was determined for both DNA strands using overlapping primers. To express yeast ODCase in E. coli, the NdeI, BamHI-ended ura3 coding sequence was isolated from a preparative agarose gel, purified using Jetsorb gel extraction reagents (Genomed), and inserted 3' to the cdd promoter of cytidine deaminase expression plasmid pCDA6022. This construction resulted in replacement of the pCDA6022 cdd gene by the ura3 gene and yielded plasmid pBGM88. ODCase expression vector pBGM88 was then introduced into E. coli SS6130 (cysB, Δwdh) by electroporation. In this strain, transcription of the plasmid-borne ura3 gene is completely derepressed (9).

ODCase was purified from E. coli SS6130(pBGM88) that had been grown at 27 °C for 16–18 h in 2× YT medium (10) supplemented with Vogel and Bonner salts (11), 1% yeast extract, ZnCl2 (10 µM), and ampicillin (150 µg/ml). The bacteria were collected by centrifugation (10,000 × g for 10 min) and suspended to 1 g of cells, wet weight/4.8 ml of buffer containing potassium phosphate (50 mM, pH 6.0), glycerol, (20%), and β-mercaptoethanol (5 mM). Cell extracts were prepared by passage of the suspension through a French press at 10,000 p.s.i. Intact cells and cell debris were removed by centrifugation at 100,000 × g for 70 min. The supernatant was filtered through a 0.2-µm filter and applied to an anion exchange column (Poros II Q, Perspective Biosystems) equilibrated with Buffer A (Tris-HCl (50 mM, pH 7.0), glycerol (5%, w/v), and β-mercaptoethanol (5 mM)). Those fractions in the column flow-through with ODCase activity were pooled and dialyzed against Buffer A for 16 h at 4 °C. The dialyzed protein solution was applied to a second anion exchange column (HR 10/10 Mono Q, Amer.

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§ The abbreviations used are: OMP, orotidine 5'-phosphate; ODCase, orotidine-5'-phosphate decarboxylase; UMP, uridine 5'-phosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; 6-CSNH2-UMP, 6-thiocarboxamidouridine 5'-phosphate; 6-CONH2-UMP, 6-carboxamidouridine 5'-phosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; HEPES, N-(2-hydroxyethyl)piperazine-N'-4-(2-hydroxyethyl)piperazine-N'-4-butanesulfonic acid.

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Zinc content, specific activity, and catalytic constants of various forms of yeast ODCase determined at 22 °C

| Enzyme                              | Zn/subunit | k_{cat} | K_{m} | Specific activity |
|-------------------------------------|------------|---------|-------|------------------|
| Native ODCase expressed in yeast    | 0.89 ± 0.16| 13 ± 1.2| 0.7   | 27.2 ± 2.5       |
| ODCase expressed in bacteria        | 0.06 ± 0.01| 19 ± 1.2| 0.6 ± 0.04| 39.3 ± 2.5      |

a Value from Ref. 2.

RESULTS AND DISCUSSION

To allow expression of yeast ODCase in E. coli, two residues of the native yeast enzyme were replaced; the penultimate N-terminal serine was changed to histidine, and the C-terminal asparagine was changed to aspartate. These replacements showed no significant effect on the activity of the bacterially produced enzyme, compared with the values obtained for the enzyme isolated from yeast (Table I).

Expression of the yeast ura3 gene in bacteria yielded 75–100 mg of highly purified ODCase per liter of culture and produced a fully active enzyme that contained less than 0.1 molar eq of zinc, as indicated by atomic absorption spectroscopy. In contrast, ODCase purified from yeast contained 0.89 mol of zinc/subunit (Table I). Analysis of concentrated samples of the bacterially produced enzyme by inductively coupled plasma emission spectroscopy (Garratt-Callahan Co., Millbrae, CA) revealed the presence of less than 0.12 molar eq of a variety of other metals including cadmium, cobalt, copper, iron, manganese, molybdenum, nickel, and lead. These results appear to invalidate the hypothesis that metals directly participate in the mechanism of OMP decarboxylation. Amino acid sequencing of the native yeast ODCase revealed that the N terminus is blocked, presumably as a result of posttranslational modifications. As expected from our previous experience with bacterial expression systems, the enzyme purified from E. coli was found to be devoid of posttranslational modifications, as judged by amino acid sequencing and electrospray mass spectrometry. SDS-polyacrylamide gel electrophoresis analysis of the yeast and bacterially generated enzymes showed that these proteins have similar electrophoretic mobilities.

On the basis of this present evidence, we do not understand why the enzymes from E. coli and from yeast, prepared by the same purification procedure, resemble each other in catalytic activity but differ in their metal content. The two-residue difference between yeast and bacterially expressed ODCase seems unlikely to be the source of the difference in metal content, because the introduced amino acids, histidine and aspartate, might be expected to assist rather than interfere with the coordination of zinc. That source of difference cannot be excluded, however, in view of the possibility that other
interactions might be affected by these replacements in such a way as to reduce the enzyme’s affinity for zinc. To determine whether there might be other observable differences between enzymes obtained from the two expression systems, we determined the susceptibilities of the yeast-expressed and bacterially expressed enzymes to thermal inactivation. Fig. 3 shows that the extent of inactivation of both enzymes exhibited a sigmoidal dependence on temperature. When the inflection points were compared, the zinc-containing enzyme isolated from yeast showed a $T_{\text{inact}}$ of 58.0 °C, whereas the zinc-deficient enzyme expressed in bacteria was markedly less stable, with a $T_{\text{inact}}$ of 46.2 °C. The extent to which these differences might arise from differences in posttranslational modification remains to be determined. Experiments designed to interconvert these species were not successful. Removal of zinc from the enzyme expressed in yeast by dialysis in the presence of EDTA (0.01 M) resulted in loss of activity as described (3). Attempts to add ZnSO$_4$ to bacterially expressed ODCase, both in the absence and presence of 2 M urea, failed to produce a zinc-ligated enzyme.

Consistent with the lack of zinc in the bacterially produced enzyme, expression of the yeast $ura3$ gene in *E. coli* yielded an enzyme that, unlike the enzyme expressed in yeast, was insensitive to inactivation by 1,3-dimercaptopropanol and EDTA. However, the enzyme expressed in bacteria remained highly sensitive to reversible competitive inhibition by 6-CSNH$_2$-UMP ($K_i = 3.5 \times 10^{-9}$ M), like the enzyme expressed in yeast. That seems surprising because the design of 6-CSNH$_2$-UMP was predicated on the possibility that zinc, with its high affinity for sulfur ligands, might be present at the active site (7). The basis of this inhibitor’s remarkable binding affinity and of the catalytic process itself remains to be determined by structural studies that are now in progress.

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