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No Metal Cofactor in Orotidine 5′-Monophosphate Decarboxylase

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Orotidine 5′-monophosphate decarboxylase (OMP decarboxylase, ODCase) is an important enzyme that catalyzes the final step of *de novo* pyrimidine nucleotide biosynthesis. The mechanism of this unique enzyme and whether metal ions play any role in catalysis have been topics of intense research interest. In this report, the role of Zn in ODCase was reexamined. Atomic absorption (AA) and X-ray absorption (XAS) spectroscopic studies did not detect zinc in active enzyme samples at high concentration. The XAS results also indicated the absence of other transition metal ions in ODCase. © 1999 Academic Press

Orotidine-5′-monophosphate decarboxylase (OMP decarboxylase, ODCase) catalyzes the decarboxylation of orotidine 5′-monophosphate (Figure 1). The enzymatic conversion of OMP to uridine 5′-monophosphate (UMP) is the final step of *de novo* pyrimidine nucleotide biosynthesis and is mechanistically distinctive. The non-enzymatic reaction has been described as the slowest biological reaction with a half-life of 78 million years (1). ODCase accelerates the reaction by a factor of 1.4×10^{17}, making it a very proficient enzyme (1). However, unlike reactions catalyzed by other decarboxylases, in which the carbanion intermediates can be stabilized by delocalizing the electron pair into an electrophilic π-system of the substrate or a covalently attached cofactor (2), the decarboxylation of OMP yields a nonconjugated carbanion intermediate and ODCase possesses no cofactors to use for covalent stabilization. Thus, the mechanism by which ODCase stabilizes the carbanion intermediate and catalyzes the decarboxylation of OMP has been a target of significant research effort but remains unknown.

Three different mechanisms have been proposed to explain the facile enzymatic decarboxylation (3-5). Model studies led Beak and Siegel (3) to suggest that the substrate is bound in the zwitterion form and that the cationic nitrogen-1 stabilizes the carbanion intermediate through dipole induction. In this zwitterion mechanism, it is envisioned that the reaction is initiated by the protonation of oxygen-2 in the substrate leading to resonance formation of a positive charge on nitrogen-1. Recently Lee and Houk (5) have proposed that proton transfer to oxygen-4 would give a further stabilized carbene-like intermediate. Recent model studies of ours have demonstrated the viability of this alternative proposal (6), while in another study, we have confirmed the presence of a discrete, carbon-6 based nucleophile as an intermediate in the model reaction, consistent with either of the above mechanisms (7). Enzymological studies have demonstrated that 1-β-D-ribofuranosylbarbiturate 5′-monophosphate (BMP), which resembles the postulated carbanion intermediate, is a very potent inhibitor of ODCase (8). This result suggests the involvement of a C-6 based carbanion as an intermediate, consistent with either the zwitterion or the carbene mechanism occurring on the enzyme. On the other hand, results from studies on substrate analogs (8, 9) such as BMP and on kinetic isotope effects (10, 11) have provided strong evidence against an alternative covalent mechanistic proposal, which involves nucleophilic attack at carbon-5 by an active site residue and protonation at carbon-6 prior to decarboxylation/elimination that would avoid the formation of the vinylic carbanion (4).

If the enzyme utilizes either the zwitterion or the carbene mechanism to catalyze the decarboxylation, it would be expected to provide a means of electrophilic stabilization (e.g., a proton source or a metal ion) adjacent to either oxygen-2 or oxygen-4 of the substrate. Intrigued by the proficiency of ODCase and the role of divalent metal ions in other decarboxylases, Miller et al. (12) have reexamined the possibility of metal ions as cofactors. They have reported that two moles of Zn are present for each mole of subunit for the dimeric yeast ODCase and that Zn is required for enzyme activity.

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They have further reported that ODCase is inactivated by EDTA and other zinc-chelating agents (12).

In our study on ODCase purified from *Saccharomyces cerevisiae* BJ5464, it has been observed that zinc-chelating agents have no effect on the catalytic activity since purification of ODCase is routinely carried out in presence of 2 mM EDTA (13). This observation is consistent with previous results on the Zn content in ODCase reported by Shostak and Jones (9). In light of the conflicting results in the literature, we have reexamined the Zn content, using atomic absorption (AA) and X-ray absorption (XAS) spectroscopy, of ODCase isolated in our laboratories.

MATERIALS AND METHODS

PGU2, a plasmid for expression of ODCase in yeast (14), was introduced into *S. cerevisiae* BJ5464 (obtained from the Yeast Genetic Stock Center at the University of California-Berkeley) by a standard transformation procedure (15). For production and purification of ODCase, procedures described by Bell and Jones (13) were used with slight modification. Specific activity of purified ODCase was 33 to 40 U/mg. Enzyme activity was assayed by monitoring the decrease in absorbance at 285 nm at 25°C in 100 mM potassium phosphate buffer, pH 6.0 using an extinction coefficient of 1650 M^{-1} cm^{-1} for the conversion of OMP to UMP (13).

Atomic absorption spectroscopy was carried out on a Unicam 929 AA spectrometer in flame mode. The detection limit is estimated to be 0.013 mg/L (0.2 μM) for Zn. X-ray absorption spectroscopy (XAS) studies were performed at the Stanford Synchrotron Radiation Laboratory (SSRL) on unfocused wiggler beamline 7-3 under dedicated conditions (3 GeV, 50-100 mA) in fluorescence mode. The sample (4.2 mM subunit concentration) was loaded in a lucite solution cell (23 $\times$ 1 $\times$ 3 mm with 37 mm Kapton window) and placed in a continuous flow liquid helium cryostat (Oxford Instruments CF1208) at 10 K. The total incoming signal was monitored on the multichannel analyzer of a 13 element Ge array detector (Canberra) with the incident energy at 10,000 eV, above the Zn K-edge at 9661 eV (16). A wide window on the detector was used to monitor the signal from all first row transition elements (4493 eV–9661 eV). The detection limit is estimated to be at micromolar levels.

RESULTS AND DISCUSSION

ODCase samples in a series of concentrations from 11 μM to 141 μM (subunit) were analyzed for Zn content using atomic absorption spectroscopy. A series of Zn solutions in phosphate buffer (100 mM) with a range of concentrations corresponding to that of ODCase samples, were used as standards. Representative results are listed in Table 1. The corresponding concentration of Zn for the ODCase sample (45 μM in subunit) used in Entry 2 would be 90 μM according to the 2 mole of Zn/mole of protein subunit stoichiometry reported by Miller et al (12). The absence of Zn in ODCase is apparent by comparing the atomic absorption data in Entries 1 and 2. Several controls were also performed to test whether protein might interfere with the atomic absorption signal. Overnight hydrolysis of ODCase in hot 6 N HCl still showed no detectable levels of Zn (entry 3), while ODCase spiked with approximately one equivalent of Zn gave a signal within error of the value obtained for the standard (compare entries 1 and 4). As one final control, Zn was readily detected in a sample of carboxypeptidase A (data not shown), an enzyme known to require Zn as a cofactor. It should be noted that the ODCase samples used in these AA experiments were fully active in the standard assay and showed no change in activity upon addition of Zn to the assay mixture.

TABLE 1

Zn Atomic Absorption of ODCase Samples

Entries	Sample	Concentration (μM)	Absorption
1	Zn in buffer	46	0.349
2	ODCase	45	0.003
3	Hydrolyzed ODCase	53	N.D.[a]
4	ODCase + Zn	53 (ODCase) 46 (Zn)	0.363

[a] Not detected. Reading (−0.002) was below that of buffer used as blank.

The above results, suggesting the absence of Zn in ODCase, were further confirmed by X-ray absorption spectroscopy (XAS). XAS analysis of a 4.2-mM, fully active ODCase sample (subunit concentration) failed to detect any Zn or significant amounts (>1% of protein concentration) of Mn, Fe, Co, Cu, or Ni. The expected Zn concentration, assuming 2 moles of Zn per mole of subunit, is 8.4 mM, which is well above the detection limit.

Our results have demonstrated that ODCase does not contain Zn or other transition metal ions as cofactors. This conclusion is consistent with a previous report by Shostak and Jones (9). At this point, we do not know why ODCase samples from different yeast strains appear to have different requirements for metal cofactors. The mechanism that ODCase employs to efficiently catalyze the decarboxylation reaction remains a topic of significant research interest.

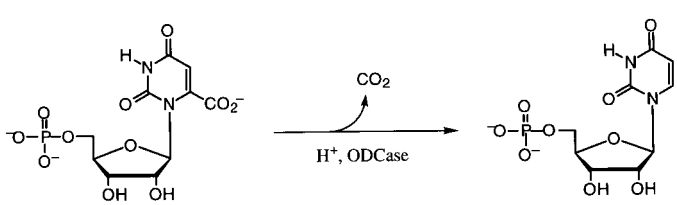

FIG. 1. The enzymatic decarboxylation of OMP to UMP.

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