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A Reexamination of the Substrate Utilization of 2-Thioorotidine-5′-monophosphate by Yeast Orotidine-5′-Monophosphate Decarboxylase

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A potential alternate substrate for orotidine-5′-monophosphate decarboxylase, 2- thio-orotidine-5′-monophosphate, was synthesized enzymatically and purified by a modification of a previous account (K. Shostak, and M. E. Jones 1992, *Biochemistry* **31,** 12155–12161). Characterization of the product was confirmed by mass spectrometry, ^{31}P NMR, and utilization by orotate phosphoribosyltransferase in the direction of pyrophosphorolysis. The previous work probably did not result in the purification of the desired compound, as evidenced by our observation of 2-thioOMP's sensitivity to high temperature, as used previously. Using a very sensitive HPLC assay for the potential decarboxylated product 2-thioUMP, no measurable activity of ODCase toward the alternate substrate was observed, representing a decarboxylation rate decreased by 10^{-7} from the k_{cat} for ODCase toward OMP. Additionally, 2-thioOMP effects no inhibition of ODCase decarboxylation of OMP at a concentration of 50 μM, indicating a poor ability to bind to the ODCase active site. The results bear implications for proposed mechanisms for catalysis by ODCase.

Recent protein crystal structures (1–4) have provided new insights into the catalytic mechanism of OMP decarboxylase (ODCase),[2] the enzyme with the highest measured catalytic proficiency (*5*). Originally, the catalytic mechanism of ODCase was presumed to proceed through a protonation step at O2 preceding decarboxylation (Fig. 1), yielding a zwitterionic intermediate, based on the observation of rates of uncatalyzed decarboxylation of model compounds (*6*). Support for this mechanism arose from enzyme inhibition (*7*) and isotope effect studies (*8–10*). A lysine residue (Lys93 of the yeast enzyme), common to all protein sequences of ODCases from many organisms, was found to be critical for catalysis and postulated as the general acid group

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[2] Abbreviations used: OMP, orotidine-5′-monophosphate; ODCase, OMP decarboxylase; 2-thioOMP, 2-thioorotidine-5′-monophosphate; Lys93, residue 93 (lysine) in the yeast ODCase sequence; OPRTase, orotate phosphoribosyltransferase; PRPP, 5-phosphorylribose-1-pyrophosphate.

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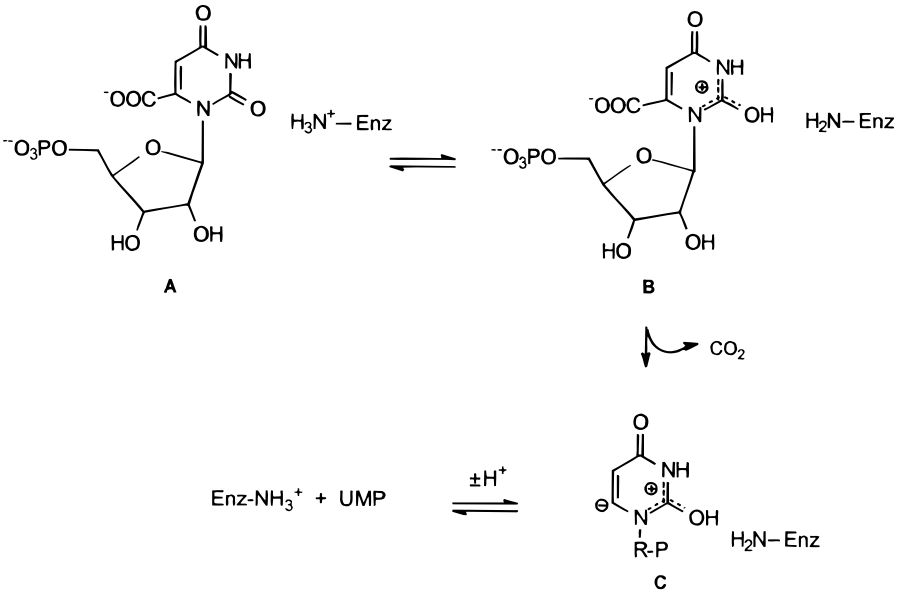

FIG. 1. Proposed mechanism of ODCase catalysis by O2 protonation. Several resonance structures are possible for distribution of charges in the intermediates; previous depictions have shown isolation of the positive charge at N1. Enz-NH_3^+, lysine side chain at active site (Lys 93 of the yeast enzyme).

protonating the substrate (*11*). Substitution of sulfur for oxygen at the 2-carbonyl position apparently resulted in a substrate analog, 2-thioorotidine-5′-monophosphate (2-thioOMP), that was incapable of undergoing catalysis (*12*).

The four crystal structures (*1–4*) showed an orientation for the inhibitors with C6, rather than C2, aligned with the invariant lysine residue. A mechanism proposed from this active-site structure (*1*,*3*,*4*) suggests that this lysine side chain donates a proton to C6 of OMP to directly replace the departing carboxylate (Fig. 2), invoking no critical involvement of the substituent at C2.

In light of the new mechanism proposed, and to extend our previous finding that 2-thioUMP has essentially the same inhibition constant as UMP (*13*), a reexamination of the substrate utilization of 2-thioOMP seemed warranted. Possibly, the enzymatic synthesis method used previously (*12*) yielded a compound other than the anticipated product 2-thioOMP. We have synthesized 2-thioOMP from 2-thioorotate, 5-phosphoryrlribose-1-pyrophosphate (PRPP) and orotate phosphoribosyltransferase (OPRTase), and devised an HPLC assay for detection of 2-thioUMP which could be generated from the decarboxylation by ODCase. We have found 2-thioOMP to be a fairly unstable compound and suspect that the compound isolated previously (*12*) was likely an undesired product of this instability. However, the 2-thioOMP we have synthesized and characterized does not undergo enzymatic decarboxylation and does not bind to ODCase with an affinity even remotely similar to that for OMP, calling again into question the role of the 2-carbonyl group of the substrate during catalysis.

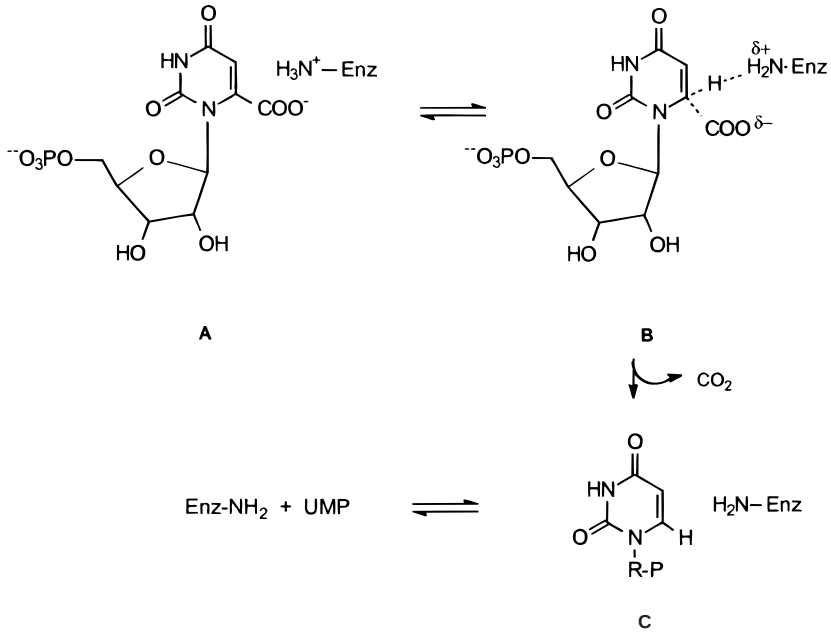

FIG. 2. Proposed mechanism of ODCase catalysis by C6 protonation. The pyrimidine ring is rotated 180° about the glycosidic bond from the structure in Fig. 1. The crystal structure of ODCase and UMP (*1*) shows proximity of the active-site lysine with C6; this feature of the active-site structure is shown in C.

MATERIALS AND METHODS

2-Thioorotate, previously purchased from Aldrich (*12*), is not currently commercially available, and was instead synthesized from thiourea (Sigma) and oxaloacetate monoethyl ester (from the diethyl ester, Sigma) according to a previous account (*14*). OPRTase was a generous provision from the laboratory of Dr. Charles Grubmeyer, Temple University. 2-Thiouracil and PRPP were from Sigma. OMP and [*carboxyl*-^{14}C]OMP were synthesized according to the modified procedure (*9*) of the original synthesis (*15*). Yeast ODCase was isolated as the predominant protein eluting from a column of Affi-Gel Blue affinity chromatography resin, as described previously (*16*).

2-thioOMP was enzymatically synthesized as described previously (*12*), with the following modifications. Instead of terminating the reaction by boiling, the reaction was quenched by the addition of ethanol to a final concentration of 50%, and precipitated protein was removed by filtration. The solution was evaporated to a small volume, and the products were separated by anion-exchange HPLC, using a Hamilton PRP-X100 column (305 × 7 mm) and the following elution profile, 0–10 min, 0–0.8 M NH_4HCO_3, pH 8.4, linear gradient; 10–25 min, isocratic 0.8 M NH_4HCO_3, pH 8.4. Products eluting from the column were detected by absorbance at 270 or 276 nm. 2-thioOMP was quantitated by absorbance at 276 nm, according to the extinction coefficient (ε_{276} = 11,000 M^{-1}) obtainable from the isosbestic point in the conversion of 2-thioorotate to 2-thioOMP demonstrated previously (*12*). NH_3 present in HPLC

fractions of 2-thioOMP was assayed using Nessler's reagent, with NH_4HCO_3 solution as a standard. 2-thioUMP was enzymatically synthesized as before (*13*), using overproduced uracil phosphoribosyltransferase from an *Escherichia coli* strain kindly provided by Dr. Jan Neuhard, University of Copenhagen. 2-thioUMP was purified under the same HPLC conditions as above.

Mass spectrometry of 2-thioOMP. An HPLC elution fraction corresponding to a major peak arising in the 2-thioOMP enzymatic synthesis was subjected to analysis on a Bruker Esquire mass spectrometer, under the following electrospray ionization (ESI) conditions: ESI voltage, 4.0 kV; direct infusion; capillary temperature, 300°C; anion detection; scan range, 50–2200 *m*/*z*; scan resolution, 0.6 *m*/*z*.

NMR analysis of 2-thioorotate and 2-thioOMP. Synthesized 2-thioorotate was analyzed by ^{13}C NMR (proton decoupled), and 2-thioOMP analyzed by ^{31}P NMR, using a Varian Gemini 400 MHz NMR spectrometer. Samples of both compounds were prepared in D_2O, with NaOD added to the 2-thioorotate to increase solubility.

OPRTase-catalyzed pyrophosphorolysis of 2-thioOMP. Further structural analysis of the suspected 2-thioOMP was assessed using the HPLC-purified material as a substrate for the pyrophosphorolysis reaction catalyzed by OPRTase. Reaction conditions were identical to the OPRTase reaction for 2-thioOMP synthesis, with the exception of 40 μM 2-thioOMP and 200 μM sodium pyrophosphate replacing 2-thioorotate and PRPP as substrates, and the exclusion of inorganic pyrophosphatase. Products were separated under the same HPLC conditions as used for the purification of 2-thioOMP.

Enzymatic assays of ODCase using OMP and 2-thioOMP. ODCase activity at saturating OMP concentration (50 μM) was measured using a spectrophotometric assay (*17*) with $\Delta\varepsilon_{286} = -2250\ M^{-1}$. Decarboxylation activity was measured in the presence of 50 mM Tris-Cl, pH 8.0. For reactions containing 200 mM NH_4HCO_3, a solution of 1 M Tris-Cl, pH 6.9, was added to give a final concentration of 50 mM Tris and a final pH of 8.0. Activity was measured within the first minute of initiating the reaction. Activities at subsaturating concentrations were measured using the ^{14}C displacement assay with [*carboxyl*-^{14}C]OMP (*18*).

Activity of ODCase toward 2-thioOMP was measured by detection of 2-thioUMP by HPLC, with the measured retention times for 2-thioOMP (10 min) and 2-thioUMP (6.8–6.9 min) under the separation conditions described above. 2-thioUMP was quantitated using $\varepsilon_{274} = 13{,}000\ M^{-1}$. The concentration of 2-thioOMP was 50 μM, and the ODCase concentration was increased to as high as 220 μg mL^{-1}. Tris-Cl was added to a final concentration of 50 mM, pH 8.0, and NH_4HCO_3 (from 2-thioOMP HPLC fractions) was 200 mM. From 1.0-mL reactions, 100-μL samples were taken for HPLC analysis. In some samples, 2-thioUMP was included as a standard.

RESULTS AND DISCUSSION

Shostak and Jones (*12*) showed that the activity of OPRTase toward 2-thioorotate and PRPP was consistent with formation of a nucleotide closely resembling OMP, from the UV absorbance spectra of the enzymatic reaction. The OPRTase used in this enzymatic synthesis has a high specificity for orotate versus other heterocycles, due to hydrogen bonding of N3–O4 and the carboxylate of the natural substrate (*19*); these strucural features of the pyrimidine substrate are unchanged in the thio analog.

The lack of activity of OPRTase toward 5-carboxyuracil (C. Grubmeyer, personal communication), for example, indicates that the exact location of the carboxylate group on the pyrimidine is critical for binding and catalysis, and formation of the expected 2-thioOMP by this enzyme is quite predictable.

Our enzymatic synthesis of 2-thioOMP produced a major peak in the HPLC profile emerging at 10 min,well before the elution of 2-thioorotate. This peak increased in intensity with length of time of the reaction and presence of inorganic pyrophosphatase to remove pyrophosphate (Fig. 3). The previous description of the enzymatic synthesis of 2-thioOMP (*12*) reported that the nucleotide emerged from anion-exchange HPLC *after* the starting compound. We thus subjected our product to further analysis.

^{13}C NMR analysis of 2-thioorotate showed peaks with the following chemical shifts and their tentative assignments: 181.7 ppm (C2), 176.2 ppm (C7), 174.9 ppm (C4), 161.1 ppm (C6), 104.0 ppm (C5). The pattern is similar to, but distinct from, the ^{13}C signals from orotate, and no discernable traces of orotate signals in this preparation were evident.

Mass spectrometry of the 10-min fraction revealed the following anionic masses: 339.0, 360.9, 383.0, and 405.0 (Fig. 4). The mass of 383.0 matches that for the monoanion of 2-thioOMP. The mass of 405.0 matches that for the monoanion of 2-thioOMP in which Na^+ has replaced one dissociable proton. The two lower masses are in agreement with the monoanion and Na^+-substituted monoanion of 2-thioUMP, suggesting decarboxylation within the conditions of the mass spectrometer. Analysis

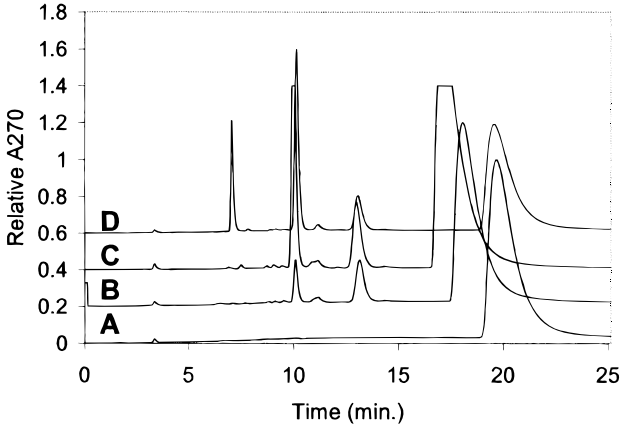

FIG. 3. HPLC traces of 2-thioOMP synthesis. Enzymatic synthesis reactions with 2-thioorotate and OPRTase are as described in Ref. (*12*), with the modifications described in the text. Chromatograms are normalized so that the highest peak within each trace is assigned an absorbance of 1.0. A: 2-Thioorotate only. B: 2-thioOMP enzymatic synthesis, 1 h at 30°C, without inorganic pyrophosphatase. C: 2-thioOMP enzymatic synthesis, 1 h at 30°C, with 10 units inorganic pyrophosphatase. D: 2-thioOMP enzymatic synthesis, 3 h at 30°C, with 10 units inorganic pyrophosphatase, with 2-thioUMP included as a reference standard. The fractions from the peaks at 10 min were collected and analyzed. The peaks in B–D at 13 min arise in control reactions without OPRTase (data not shown), and are presumed to be indicative of a contaminant in the PRPP.