Bioorganic Chemistry **27,** 297–306 (1999)
Article ID bioo.1999.1140, available online at http://www.idealibrary.com on IDEAL®

Active Site Probes for Yeast OMP Decarboxylase: Inhibition Constants of UMP and Thio-Substituted UMP Analogues and Greatly Reduced Activity toward CMP-6-Carboxylate

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Received November 6, 1998

The catalytic mechanism of orotidine-5′-monophosphate decarboxylase (ODCase, EC 4.1.1.23) involves a proton-sensitive step, probably proton donation to one of the carbonyl oxygens of the substrate, and may also include participation of a Zn^{2+} ion. To probe the active site for these mechanistic features, thio-substituted analogues of the product UMP were used as inhibitors of yeast ODCase. The intrinsic inhibition constants of the anionic pyrimidines were calculated using the measured inhibition constants and the pK_a values of the respective compounds. 4-ThioUMP is a stronger inhibitor than UMP, while 2-thioUMP has a K_i virtually the same as that for UMP. A potential alternate substrate, CMP-6-carboxylate, has been synthesized and found to have undetectable activity and weak binding to ODCase. The results are discussed in a unified model for catalysis involving protonation at O2 and a proposed Zn^{2+} interaction at O4.

Since the observation that the catalytic proficiency of yeast orotidine-5′-monophosphate decarboxylase (ODCase, EC 4.1.1.23) is higher than that measured for any other enzyme (*1*), the mechanism of decarboxylation has attracted heightened interest. Beak and Siegel (*2*) originally proposed a mechanism in which the enzyme protonates the substrate orotidine-5′-monophosphate (OMP) at O2, leading to formation of a zwitterionic intermediate more capable of undergoing decarboxylation. Smiley *et al.* (*3*) observed evidence for a proton-sensitive, noncovalent mechanism, consistent with the proposal of Beak and Siegel, in the changes in carbon isotope effects with varying pH. Levine *et al.* (*4*) measured inhibition constants for UMP (Fig. 1) and 6-azaUMP and deduced from the pK_a values of the nitrogenous rings that ODCase was likely binding the anionic forms (referred to herein as "UMP anion") of these ligands preferentially. Smiley and Jones (*5*) identified a cationic amino acid residue, Lys93 of the yeast enzyme, that was instrumental in both catalysis and the preference for anionic ligands.

Shostak and Jones (*6*) observed no catalytic activity of ODCase toward 2-thioOMP,

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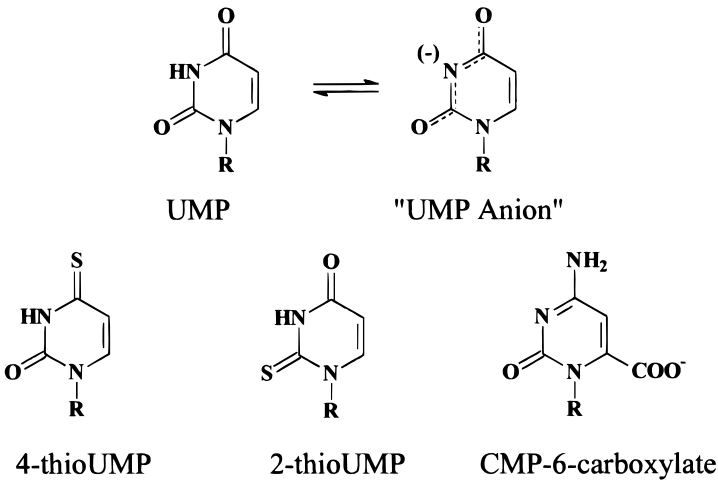

FIG. 1. Structures for UMP and dissociated anionic form (UMP anion), thio-substituted analogues 4-thioUMP and 2-thioUMP, and CMP-6-carboxylate. R, ribose 5′-phosphate.

although this analogue was found to bind reasonably well by virtue of its inhibition of ODCase activity toward OMP. In contrast, 4-thioOMP was found to be a substrate with kinetic parameters similar to those for OMP. However, the authors offered no mechanistic rationale for these observations. Although the thiocarbonyl sulfur would be expected to be a weaker base than the carbonyl oxygen, and activity would be expected to decrease in a mechanism involving O2 protonation, the complete disappearance of measurable activity toward 2-thioOMP would not necessarily be anticipated.

More recently, additional mechanistic details have been proposed. Cleland and Kreevoy (*7*) suggested that ODCase might catalyze decarboxylation concerted with proton transfer and that the proton transfer event might manifest itself as a short, strong hydrogen bond in the interaction of the enzyme with the transition state. Lee and Houk (*8*) proposed that O4 of the substrate would be a more likely site for protonation, leading to a decarboxylated carbene intermediate. Miller *et al.* (*9*) found zinc in extensively dialyzed enzyme and observed loss of catalytic activity upon treatment of the enzyme with Zn^{2+} chelators.

Sulfur substitution for carbonyl oxygens on the uracil ring of UMP, and measurement of the inhibition constants of these analogues, was designed in this study to provide more information on the types of contacts between ODCase and pyrimidine nucleotide ligands. Sulfur substitution at a position which is involved in a hydrogen bond contact would be expected to result in a thioUMP analogue with a weaker binding affinity (*10*). If hydrogen bond contact between the enzyme and the substrate occurs only in the transition state, as proposed by Cleland and Kreevoy (*7*), then sulfur substitution might be inconsequential to the binding affinity of thioUMP analogues, since UMP, the product of the decarboxylation, is a ground-state inhibitor. Another possibility is that thio-substitution might lead to a tighter binding affinity, if the carbonyl oxygen undergoing substitution is contacted by the Zn^{2+} found by Miller *et al.* (*9*). We thus

sought to illuminate further the details of the ODCase active site by measuring the inhibition constants for UMP and thio-substituted analogues 4-thioUMP and 2-thioUMP (Fig. 1) and calculating the inhibition constants for the respective anionic forms, according to the model for inhibition of Levine *et al.* (*4*) Additionally, we synthesized CMP-6-carboxylate (Fig. 1), a potential alternate substrate, and attempted to measure ODCase-catalyzed decarboxylation of this compound.

MATERIALS AND METHODS

UMP and thio-substituted analogues. UMP and 4-thioUMP were purchased from Sigma and used without purification. 2-ThioUMP was synthesized enzymatically (*11*) using 2-thiouracil and 5-phosphorylribose 1-pyrophosphate (Sigma Chemical Co.) and recombinant *Bacillus caldolyticus* uracil phosphoribosyltransferase produced in *Escherichia coli* (*12*). In 5-mL reactions, conversion of 5 μmol of 2-thiouracil to 2-thioUMP was complete after 20 min using published conditions (*12*), as judged by the disappearance of 2-thiouracil on silica gel TLC, using 19:1 CH_2Cl_2:MeOH (R_f 2-thiouracil = 0.3; R_f 2-thioUMP = 0). Protein was precipitated from the reaction mixture by addition of EtOH to a final concentration of 50%, the supernatant was evaporated to 1 mL, the pH was adjusted to 7.0 with HCl, and water was added to give a final concentration of 1.7 mM as determined from the extinction coefficient, ε_{274}, 13,000 M^{-1} cm^{-1}. The product displayed a UV absorbance maximum of 274 nm at pH 7.0, showed a single spot on PEI-cellulose TLC (*13*), and was used without further purification.

Purification of yeast ODCase. Yeast ODCase was produced from strain BJ5424 (Yeast Genetic Stock Center, University of California, Berkeley) carrying plasmid pGU2 (*14*). Following induction of yeast cultures with galactose and cell lysis, the enzyme was purified using $(NH_4)_2SO_4$ fractionation and Affi-Gel Blue chromatography essentially as described (*15*). An additional purification step was employed: Affi-Gel Blue chromatography fractions containing ODCase were pooled, concentrated, and chromatographed on a MonoQ anion exchange column using the Pharmacia FPLC system. ODCase emerged at the beginning of a 0–200 mM NaCl gradient, with 10 mM potassium phosphate, pH 7.5, 10% glycerol, and 5 mM β-mercaptoethanol present in both gradient buffers. The enzyme solution was dialyzed extensively against multiple changes of buffer (*14*) to remove traces of 6-azaUMP and phosphate used in the purification steps. ODCase used in these experiments under our assay conditions showed a specific activity of 20 nmol min^{-1} μg^{-1} (cf. *15*).

Synthesis of CMP-6-carboxylate. The method for the synthesis of cytidine-6-carboxylate (*16*) was adapted for the synthesis of the corresponding nucleotide. CMP (Sigma) was converted to the desired compound in five steps:(i) bromination with Br_2 in pyridine/acetic acid to give 5-bromoCMP, (ii) cyano substitution with NaCN in DMSO to give 6-cyanocytidine, (iii) methanolysis with sodium methoxide in MeOH to give the methylcarboximidate, (iv) acid hydrolysis in aqueous HCl to give the methyl ester, and (v) base-catalyzed hydrolysis of the methyl ester to give the carboxylate. The final product was purified from unreacted synthetic intermediates using anion-exchange chromatography, utilizing the additional negative charge on the carboxylate at neutral pH and a retention time longer than that for any of the intermediates. From a Dowex AG 1-X8 (Bio-Rad) column equilibrated with 0.1 M NH_4CO_3, pH 8.0, the

latest UV-absorbing fractions from a gradient of 0.1–0.5 M NH_4HCO_3, representing the bulk of the UV-absorbing material, were pooled and evaporated to dryness. Two UV-absorbance spectra of this material in Tris, pH 7.8, and 50 mM HCl showed peaks at wavelengths of 274 and 284 nm, respectively, matching the values for the nucleoside (*16*). The ^{1}H NMR spectrum in D_2O, obtained on a Varian Gemini 2000 400 MHz spectrometer, showed one major singlet at 5.70 ppm (H-5), and one major doublet at 5.29 ppm (H-1′), corresponding to the values of 5.92 and 5.62 ppm in DMSO-d_6 for the nucleoside (*16*). The ^{13}C NMR spectrum of the synthesized compound in DMSO-d_6 showed two signals at 166 and 164 ppm, indicative of C4 (present in CMP and related compounds) and the carboxylate carbon.

Ionization constants for thio-substituted UMP analogues. The pK_a for 4-thioUMP was measured by following the decrease in absorbance at 332 nm with increasing pH. The pK_a for 2-thioUMP was measured by following the increase in absorbance at 240 nm with increasing pH. Each nucleotide (20 μM) was dissolved in one of the following buffers at 20 mM: acetate (pH 4.4–6.2), Mops (pH 7.4), Tris–Cl (7.6–8.6), glycine (pH 9.1–10.0), phosphate (11.1–11.9). The plots of absorbance values fit theoretical titration curves using pK_a values of 8.6 for 4-thioUMP and 9.2 for 2-thioUMP (data not shown).

Enzyme assays. ODCase activity was determined in the presence of varying inhibitor concentrations using the $^{14}CO_2$ displacement assay (*17*). Reactions (500 μL) were carried out at 25°C with 10 mM Mops, pH 7.0, 0.5–2.0 μM OMP, and inhibitors at concentrations estimated from preliminary assays to be near the K_i value: [UMP] = 0, 100, 200, or 400 μM; [4-thioUMP] = 0, 1.1, 2.2, or 4.3 μM; [2-thioUMP] = 0, 31, 61, or 92 μM. ODCase was diluted in 50 mM Tris–Cl, pH 7.4/10% glycerol immediately before assay, so that an amount of activity to convert <30% of substrate to product in 30 s could be dispensed in 5 μL. For competitive inhibitors, K_i values were determined from replot of the apparent K_m values versus the corresponding inhibitor concentrations; these values are reported as "Apparent K_i" in Table 1. For 2-thioUMP, K_i values were determined from replot of the slopes versus the corresponding inhibitor concentrations. The values for "Intrinsic K_i" are calculated from the proportions of ionized pyrimidines present in solution at pH 7.0, based on the pK_a values for each inhibitor.

Spectrophotometric assay for CMP-6-carboxylate decarboxylation. The spectral

TABLE 1

Inhibition Constants for UMP and Thio-Substituted Analogues with ODCase

Inhibitor	pK_a	Apparent K_i	K_i for anionic form[a]
UMP	9.5	$(92 \pm 2) \times 10^{-6}$ M	$(2.9 \pm 0.1) \times 10^{-7}$ M
4-ThioUMP	8.6	$(1.5 \pm 0.5) \times 10^{-6}$ M	$(0.38 \pm 0.13) \times 10^{-7}$ M
2-ThioUMP	9.2	$(43 \pm 7) \times 10^{-6}$ M	$(2.7 \pm 0.7) \times 10^{-7}$ M

Note. Constants were measured at 25°C, in 10 mM Mops buffer, pH 7.0, as described in the text. Each value of apparent K_i is an average of two independent determinations, with average deviation given.

[a] Calculated dissociation constants for the anionic forms of each inhibitor, according to the model for inhibition of Levine *et al.* (*4*).

difference at neutral pH between CMP (λ_{max} 271 nm; ε_{271} 9100 cm^{-1} M^{-1}) and CMP-6-carboxylate [λ_{max} 274 nm; ε_{274} 6940 cm^{-1} M^{-1}, from value for nucleoside (*16*)] allows the potential for a spectrophotometric assay for the conversion of CMP-6-carboxylate to CMP. Using equimolar solutions of both nucleotides, the spectral difference was found to be greatest at 270 nm, where $\Delta\varepsilon_{270}$ 2300 cm^{-1} M^{-1}. This positive value indicates an increase in the absorbance upon conversion of CMP-6-carboxylate to CMP. The rate of enzymatic decarboxylation was determined by recording the spectrophotometric changes upon addition of 120 μM CMP-6-carboxylate to a reaction mixture containing 25 mM Tris, pH 7.8, and 29 μg purified ODCase.

RESULTS AND DISCUSSION

Using our assay conditions for yeast ODCase, we consistently obtained a K_m value for OMP of 0.7 μM, identical to that measured by Bell and Jones (*15*). These data are represented in the zero inhibitor concentration points in Figs. 2 and 3. Competitive inhibition of ODCase by UMP was observed by Levine *et al.* (*4*) and reproduced in this study (Fig. 2), as judged by 1/*v* versus 1/[S] plots at varying concentrations of UMP that intersect at the 1/*v* axis (*18*). Our calculated value of 2.9×10^{-7} M for the K_i for UMP anion agrees reasonably well with the previous value of 1.5×10^{-7} M, with the difference likely due to changes in temperature and reaction buffer.

4-ThioUMP also displayed competitive inhibition, as seen from intersecting 1/*v*

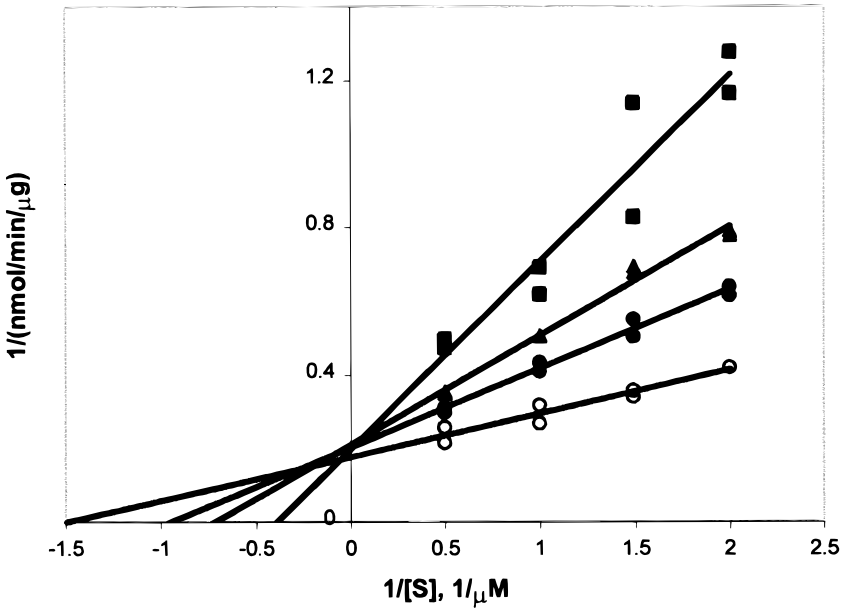

FIG. 2. Representative kinetic plot for inhibition by UMP. Assays, described under Materials and Methods, include UMP at concentrations of zero (○), 100 μM (●), 200 μM (▲), and 400 μM (■). The K_i value is determined from replot of the apparent K_m for each respective inhibitor concentration (*18*). The value listed in Table 1 is an average of two independent determinations.