Orotidine-5′-monophosphate Decarboxylase Catalysis: Kinetic Isotope Effects and the State of Hybridization of a Bound Transition-State Analogue†

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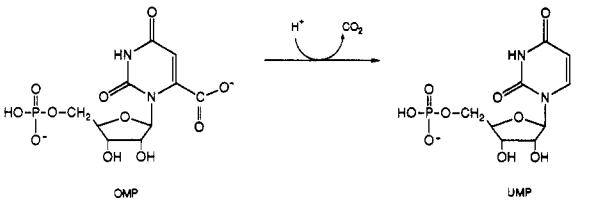
ABSTRACT: The enzymatic decarboxylation of orotidine 5′-monophosphate may proceed by an addition–elimination mechanism involving a covalently bound intermediate or by elimination of CO_2 to generate a nitrogen ylide. In an attempt to distinguish between these two alternatives, 1-(phosphoribosyl)barbituric acid was synthesized with ^{13}C at the 5-position. Interaction of this potential transition-state analogue inhibitor with yeast orotidine-5′-monophosphate decarboxylase resulted in a small (0.6 ppm) downfield displacement of the C-5 resonance, indicating no rehybridization of the kind that might have been expected to accompany 5,6-addition of an enzyme nucleophile. When the substrate orotidine 5′-monophosphate was synthesized with deuterium at C-5, no significant change in k_{cat} (H/D = 0.99 ± 0.06) or k_{cat}/K_M (H/D = 1.00 ± 0.06) was found to result, suggesting that C-5 does not undergo significant changes in geometry before or during the step that determines the rate of the catalytic process. These results are consistent with a nitrogen ylide mechanism and offer no support for the intervention of covalently bound intermediates in the catalytic process.

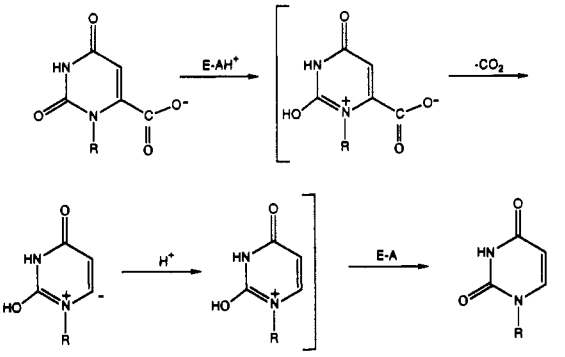
Orotidine-5′-monophosphate decarboxylase (ODCase, EC 4.1.1.23)[1] catalyzes the final step in the biosynthesis of pyrimidine nucleotides, to generate uridine 5′-monophosphate (Scheme I). Unlike many enzymes catalyzing decarboxylations, ODCase has no known requirement for a cofactor or metal, and its detailed mechanism of action remains to be established. On the basis of studies of model reactions, Beak and Siegel (1976) suggested that the reaction catalyzed by ODCase might proceed through a nitrogen ylide intermediate as shown in Scheme II. Levine et al. (1980) observed that 1-(phosphoribosyl)barbituric acid (BMP) was a very strong inhibitor of ODCase with a K_i of $\sim 10^{-11}$ M and in order to account for this fact suggested that this compound might act as a transition-state analogue because of its resemblance to the nitrogen ylide intermediate that Beak and Siegel had proposed. On the basis of studies of another model reaction, Silverman and Groziak (1982) proposed an alternative mechanism involving addition and elimination of an enzyme nucleophile as shown in Scheme III.

Formation by the enzyme of a covalently bound adduct (with either the substrate or the inhibitor) would result in a change in geometry at C-5 from trigonal (sp^2) to tetrahedral (sp^3), as shown in Scheme III. In the nitrogen ylide mechanism, however, C-5 remains trigonal throughout the catalytic sequence (Scheme II). In an effort to distinguish between these two possibilities, we synthesized BMP enriched in ^{13}C at C-5 of the pyrimidine ring and examined its interaction with ODCase by NMR. As an additional test for a change in geometry at C-5, we synthesized OMP with deuterium substituted for hydrogen at C-5 and determined the effect of this substitution on the rate constants for enzymatic decarboxylation.

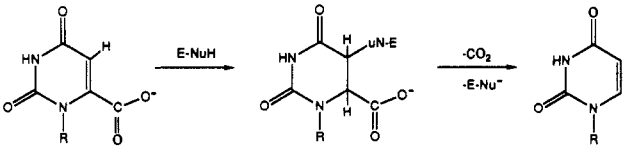
Scheme I: Reaction Catalyzed by ODCase



Scheme II: Nitrogen Ylide Mechanism, Reactions within Brackets Occurring at the Enzyme Active Site



Scheme III: Addition–Elimination Mechanism



Materials and Methods

Materials. Orotic acid, 5-phosphorylribose 1-pyrophosphate (PRPP), acetyl 2,3,5-tribenzoyl-β-D-ribofuranoside, and OMP pyrophosphorylase was obtained from Sigma Chemical Co. Diethyl [2-^{13}C]malonate was purchased from Isotec Inc. Deuterium oxide (99.8% D) was from Aldrich Chemical Co. 6-Azauridine 5′-monophosphate (AzaUMP) was synthesized

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[1] Abbreviations: ODCase, orotidine-5′-monophosphate decarboxylase; OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; BMP, 1-(5′-phospho-β-D-ribofuranosyl)barbituric acid; AzaUMP, 6-azauridine 5′-monophosphate; PRPP, 5-phospho-α-D-ribose 1-diphosphate.

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according to a published procedure (Brody & Westheimer, 1979). 1-(5′-Phospho-β-D-ribofuranosyl)[5-^{13}C]barbituric acid ([5-^{13}C]BMP) was prepared by a modification of the procedure of Levine et al. (1980).

General Methods. UV spectra and kinetic assays were recorded on a diode array spectrophotometer (Hewlett-Packard 8452A) and ^{1}H NMR spectra on a 200-MHz spectrometer (Bruker). ^{1}H NMR chemical shifts are reported in parts per million (ppm) and internally referenced to the residual HDO resonance, which was given a value of 4.7 ppm relative to tetramethylsilane. Thin-layer chromatography was carried out on silica gel and cellulose plates (Eastman) containing a fluorescent indicator, and the results were visualized with short-wavelength UV light. Silica gel plates were developed in 1-butanol saturated with water (system A), and cellulose plates were developed in 2-propanol/water/concentrated ammonium hydroxide (65:25:10, system B). Semipreparative HPLC was carried out on a strong-anion-exchange column (Whatman Partisil 10 SAX, 500 × 9.4 mm) at a flow rate of 4.0 mL/min, eluting with sodium phosphate (100 mM, pH 3.5). Analytical HPLC was carried out on a reverse-phase column (Whatman Partisil 10 ODS-2, 250 × 2.5 mm) at a flow rate of 1.5 mL/min, with a linear gradient of 0–75% methanol over 30 min. The aqueous phase contained sodium phosphate (50 mM, pH 3.5), tetrabutylammonium phosphate (10 mM), and methanol (10% v/v).

Orotidine-5′-monophosphate decarboxylase was prepared from yeast strain 15C cells [α *leu*2-3, 112 *ura*3-52 *his*4-580 *trp*1 Δ *pep*4-3] transformed with plasmid pGU2 which carries the *ura* 3 gene for ODCase (Lue et al., 1987). The yeast strains 15C and pGU2 were kind gifts from Andrew R. Buchman in the laboratory of Roger Kornberg, Stanford University. ODCase was purified by procedures that use affinity chromatography on Affigel Blue (Lue et al., 1987) and CM-cellulose (Brody & Westheimer, 1979) with a few modifications (Bell and Jones, unpublished results) including the use of EDTA in all buffers in addition to the protease inhibitors used by Lue et al. (1987). AzaUMP, used for elution of ODCase from the affinity column, was removed by exhaustive dialysis of ODCase against UMP, followed by inorganic phosphate as described by Floyd and Jones (1985). The specific activity of ODCase was routinely measured at 35–40 units/mg at 25 °C in sodium phosphate (50 mM, pH 6.0) that contained 2-mercaptoethanol (5.0 mM).

[5-^{13}C]Barbituric Acid was prepared by a modification of the procedure of Dickey and Gray (1943). Finely cut sodium (100 mg, 4.35 mmol) was dissolved in absolute ethanol (10 mL). To this solution was added diethyl [2-^{13}C]malonate (480 mg, 3.00 mmol) followed by dry urea (180 mg, 3.00 mmol) dissolved in hot absolute ethanol (10 mL, 70 °C). The solution was refluxed under nitrogen for 2 h in an oil bath at 110 °C. After cooling, the ethanol was removed under vacuum. The resulting solid was taken up in water (20 mL) and passed through a column (20 mL) containing cation-exchange resin (Dowex 50W-X8, H^+ form). After the column had been washed with additional water (40 mL), the eluate and washings were reduced in volume (~10 mL) under vacuum. Upon cooling the dihydrate of [5-^{13}C]barbituric acid crystallized from solution. After drying in an oven (110 °C, 4 h) 208 mg (1.61 mmol, 54%) of anhydrous [5-^{13}C]barbituric acid was obtained: mp 248 °C, lit. mp for unlabeled compound 250 °C (Wood & Anderson, 1909); ^{13}C NMR (H_2O, pH 6.0, external D_2O lock) δ 77.4 (5-^{13}C), (H_2O, pH 2.0, external D_2O lock) δ 38.5 (5-^{13}C), lit. δ (DMSO) 39.1 (Okada & Esaki, 1973).

2,4,6-Tris(trimethylsilyl)[5-^{13}C]barbituric acid was prepared according to the procedure of Levine et al. (1980) except that trichloromethylsilane was omitted.

1-(2′,3′,5′-Tribenzoyl-β-D-ribofuranosyl)barbituric acid was prepared according to the procedure of Levine et al. (1980). Starting with [5-^{13}C]barbituric acid (100 mg, 0.77 mmol), 418 mg (0.73 mmol) of the title compound was obtained: R_f 0.95 in system B, 0.36 in system A; ^{1}H NMR ($CDCl_3$) δ 3.8 (s, 5-H), 4.4–4.9 (m, 4′-H, 5′-H), 6.1 (m, 2′-H, 3′-H), 6.5 (s, 1′-H), 7.2–8.2 (m, 3 × C_6H_5).

1-β-D-Ribofuranosyl[5-^{13}C]barbituric acid was prepared according to the procedure of Levine et al. (1980) except that the yellow solid obtained after lyophilization was not recrystallized: R_f 0.63 in system B, 0.05 in system A; UV λ_{max} 261 (pH 7.0); ^{1}H NMR (D_2O) δ 3.4–3.9 (m, 4′-H, 5′-H), 4.3 (t, 3′-H), 4.6 (m, 2′-H), 6.1 (d, 1′-H); ^{13}C-NMR (D_2O, pH 2.0) δ 39.2 (5-^{13}C).

1-(5′-Phospho-β-D-ribofuranosyl)[5-^{13}C]barbituric Acid. 1-β-D-Ribofuranosyl[5-^{13}C]barbituric acid (10 mg, 38.5 μmol) was added to a solution of trimethyl phosphate (1.0 mL) and phosphoryl chloride (0.1 mL) at 4 °C and stirred for 4 h. The solution was added slowly to saturated sodium bicarbonate (3.0 mL) and extracted with methylene chloride (1.0 mL) to remove trimethyl phosphate. The aqueous layer was treated batchwise with cation-exchange resin (Dowex 50W-X8; H^+ form, ~100 mg) and applied on a column (250 × 25 mm) containing anion-exchange resin (Pharmacia DEAE-Sephacel). The column was eluted with a linear gradient of ammonium bicarbonate (1500 mL, 0–0.5 M, pH 7.3); the nucleotide emerged at a salt concentration of 0.18–0.2 M. Desalting by repeated evaporation yielded 4.7 mg of solid (12.5 μmol, 32 %): R_f 0.2 in system B; λ_{max} 261 (pH 7.0); ^{1}H NMR (D_2O) δ 3.7–4.1 (m, 3 H, 4′-H, 5′-H), 4.3 (t, 1 H, 3′-H), 4.6 (m, 1 H, 2′-H) 6.1 (d, 1 H, 1′-H); ^{13}C NMR (H_2O, pH 6.0, external D_2O lock) δ 77.9 (5-^{13}C). Spectrophotometric titration in lactate/NaCl buffer (0.1 M/1.0 M) gave a pK_a of 3.8; HPLC analysis indicated >95% purity and identical retention time with a commercial preparation of the unlabeled compound.

[5-^{2}H]Orotic acid was prepared by modification of a published procedure (Pascal & Walsh, 1984). Acetyl chloride (22.5 mL) was added slowly via a dropping funnel to a cooled stirred solution (4 °C) of D_2O (15 mL). Orotic acid (300 mg, 1.92 mmol) was added to the solution, and the solution was refluxed for 4 days under nitrogen. The solution was hot filtered through a glass frit funnel and the deuterated orotic acid crystallized from the filtrate upon cooling. The crystals were washed extensively with cold water, yielding 194 mg (1.23 mmol, 64%) of deuterated orotic acid: mp 345 °C; lit. mp 345–346 °C for unlabeled orotic acid (Bachstez, 1930). Deuterium incorporation was estimated as >90% by comparison of the integrated intensities of ^{1}H NMR spectra for equimolar samples of labeled and unlabeled orotic acid in D_2O containing a DMSO internal standard.

[5-^{2}H]OMP and OMP. [5-^{2}H]Orotic acid (4.7 mg, 30 μmol), PRPP (Na^+ salt) (12.8 mg, 30 μmol), magnesium sulfate heptahydrate (7.4 mg, 30 μmol), and 50 units of OMP pyrophosphorylase were added to Tris-HCl buffer (10 mL, 50 mM, pH 8.0) and allowed to stand at room temperature for 4 h. Ethanol (5 mL) was added to the solution and the solution heated (40 °C) for 30 min. The denatured protein was removed by centrifugation and the solution reduced in volume (2 mL) under vacuum. [5-^{2}H]OMP was separated from unreacted starting materials by semipreparative HPLC. OMP was prepared in the same manner. For measurement of the isotope effect, solutions containing the labeled and

unlabeled nucleotides were adjusted to the same nucleotide and sodium phosphate (50 mM, pH 6.0) concentration by appropriate dilution. ^{1}H NMR confirmed that little or no exchange of deuterium had occurred during the synthesis of [5-^{2}H]OMP.

Kinetic Measurements. Decarboxylation of OMP and [5-^{2}H]OMP to product was followed spectrophotometrically at 285 nm according to the method of Lieberman et al. (1955). All assays were performed in 1.0-cm path-length quartz cuvettes thermostated at 25.0 ± 0.1 °C. A typical assay involved thermal equilibration of buffer containing inhibitor and substrate (960 μL) for 5 min followed by injection of enzyme (40 μL) to initiate the reaction. Initial velocities were calculated from least-squares analysis of data acquired during the first 10–20% of reaction. First-order rate constants (V_{max}/K_{app}) were measured at substrate concentrations of 6×10^{-5} M ($K_M = 1.5 \times 10^{-6}$ M; Levine et al., 1978) and an AzaUMP concentration of 2.6×10^{-4} M ($K_i = 5 \times 10^{-7}$ M; Handschumacher, 1960). First-order rate constants were calculated from least-squares fits of data (Wentworth, 1965) to eq 1, where

$$A_t = (A_0 - A_\infty)e^{-kt} + A_\infty \quad (1)$$

k is the first-order rate constant (V_{max}/K_{app}) and A_t, A_0, and A_∞ are the absorbances at times t, 0, and ∞, respectively. Rate constants were calculated from data acquired for three or more half-times of reaction.

NMR Spectroscopy. ^{13}C NMR spectra were obtained on a Varian XL-400 spectrometer operated at 100.6 MHz or a GE 500 spectrometer operated at 125.8 MHz with decoupling. A 10-mm multinuclear probe and a 36° pulse with a 1.6-s recycle time were used. The sample temperature was maintained at 25 °C. Purified ODCase (29.5 mg, 0.93 μmol) was suspended in 1.83 mL of sodium phosphate buffer (50 mM, pH 6.0) that contained acetonitrile (0.1 M, internal chemical shift reference), glycerol (~0.2 M), and 2-mercaptoethanol (5 mM). Successive aliquots (1 × 35 μL, 1 × 34 μL, and 1 × 68 μL) of [5-^{13}C]BMP (13.6 mM) in water were added to the enzyme sample. Chemical shifts were internally referenced to the acetonitrile cyano resonance, which was assigned a value of 119.1 ppm relative to tetramethylsilane.

Results

^{13}C NMR of 1-(5'-Phospho-β-D-ribofuranosyl)[5-^{13}C]-barbituric Acid Free and Bound to ODCase. Figure 1, panel A, shows the spectrum of ODCase (0.51 mM in protein monomer) in the prescence of glycerol, mercaptoethanol, and acetonitrile (internal reference). Panel B shows that the spectrum of inhibitor (0.50 mM) plus ODCase (0.50 mM in protein monomer). A new broad resonance (versus panel A), centered at 78.5 ppm, appears in the spectrum. This broad resonance is interpreted to arise from the bound form of the inhibitor with ODCase. Panel C shows the spectrum of inhibitor (0.94 mM) plus ODCase (0.47 mM in protein monomer). The sharp resonance at 77.9 ppm appearing in this spectrum is the same as that for inhibitor free in solution. Panel D shows the expansion of the region from 75 to 82 ppm in panel C. The downfield shoulder due to the bound form of BMP is evident in this spectrum. In the absence of BMP, ODCase retains full activity over the time period during which the NMR experiment was carried out.

Levine et al. (1980) reported a 100% inhibition of ODCase activity at a molar ratio of inhibitor to protein monomer of 1:1. In separate experiments, at an enzyme concentration similar to that used in the NMR experiment, we observed that ODCase activity was inhibited 53% at a molar ratio of inhibitor to protein monomer of 0.5:1, and this did not change over a period of 24 h. Our results are therefore consistent with those of Levine et al. (1980).

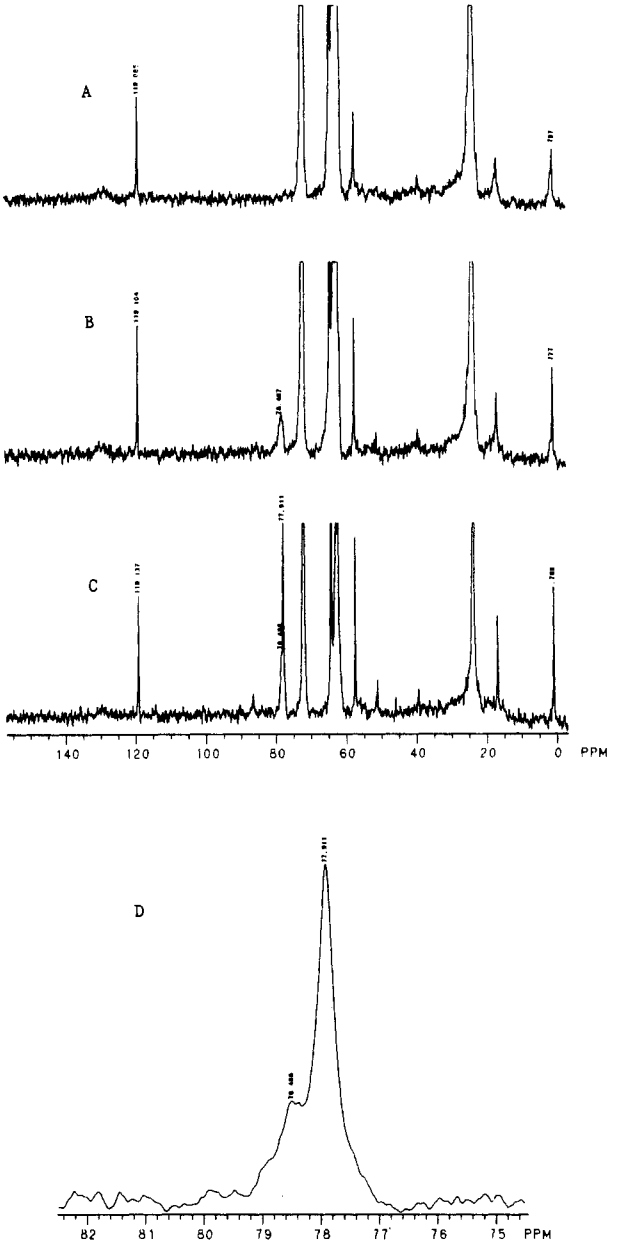


FIGURE 1: ^{13}C NMR of ODCase and ODCase–BMP complex. (Panel A) ODCase (0.51 mM in protein monomer). (Panel B) ODCase (0.50 mM in protein monomer) after addition of [5-^{13}C]BMP (0.50 mM). (Panel C) ODCase (0.47 mM in protein monomer) after further addition of [5-^{13}C]BMP (0.94 mM). Panel D shows an expansion of the region from 75 to 82 ppm in panel C. All spectra were 7200 scans. The signals at 62.7 and 72.5 ppm are due to glycerol, the signals at 24.3 and 64.3 ppm are due to 2-mercaptoethanol, and the signals at 0.7 and 119.1 ppm are due to acetonitrile.

Kinetic Isotope Effects. Initial rates (V_i) of decarboxylation were determined at high substrate concentrations relative to K_M for both OMP and [5-^{2}H]OMP. No significant difference was found ($V_i^H/V_i^D = 0.99 \pm 0.06$, $n = 17$). Because of the small extinction coefficient difference associated with the spectrophotometric assay and the low K_M value of the substrate, first-order rate constants (V_{max}/K_M) could not be determined. However, by inclusion of the competitive inhibitor AzaUMP in the assay, the first-order rate constant V_{max}/K_{app} could be determined with precision. Thus, first-order rate constants were determined at low substrate concentrations relative to K_{app} for both OMP and [5-^{2}H]OMP. As in the initial rate measurements, no significant difference was found

Table I: Secondary Kinetic Isotope Effects for ODCase

	$10^7 V_i$ (M s^{-1})				$10^4(V_{max}/K_{app})$ (s^{-1})		
	V_2^H	V_2^D	V_2^H/V_2^D		k_H	k_D	k_H/k_D
1[a]	1.57	1.57	1.00	1[d]	11.1	10.8	1.03
	1.52	1.54	0.99		10.5	11.4	0.92
	1.56	1.64	0.95		11.2	10.2	1.10
	1.56	1.48	1.05		10.9	10.9	1.00
	1.58	1.58	1.00		10.0	11.0	0.91
2[b]	1.70	1.63	1.04	2[e]	11.5	11.7	0.98
	1.53	1.75	0.87		11.7	11.7	1.00
	1.77	1.62	1.09		11.8	11.7	1.01
	1.63	1.63	1.00		11.2	11.3	0.99
	1.67	1.78	0.94		11.1	10.4	1.07
	1.66	1.76	0.94	mean			1.00
	1.65	1.71	0.96	SB			±0.06
3[c]	0.54	0.53	1.02				
	0.53	0.55	0.96				
	0.55	0.57	0.96				
	0.58	0.53	1.09				
	0.58	0.56	1.04				
mean			0.99				
SB			±0.06				

[a] Reactions were conducted at 25.0 ± 0.1 °C in 50 mM sodium phosphate–75 μM sodium citrate, pH 6.0, that contained 62 μM OMP or [5-^{2}H]OMP, 12.8 μg/mL protein, and 3.8 mM 2-mercaptoethanol. [b] Same as footnote *a* except that OMP and [5-^{2}H]OMP were 122 μM and 2-mercaptoethanol was 2.6 mM. [c] Same as footnote *a* except that protein was 10.5 μg/mL and 2-mercaptoethanol was 5.0 mM. [d] Reactions were conducted at 25.0 ± 0.1 °C in 140 mM sodium phosphate–500 μM sodium citrate, pH 6.0, that contained 62 μM OMP or [5-^{2}H]OMP, 260 μM AzaUMP, 118 μg/mL protein, and 4.6 mM 2-mercaptoethanol. [e] Same as footnote *d* except OMP and [5-^{2}H]OMP were 92 μM.

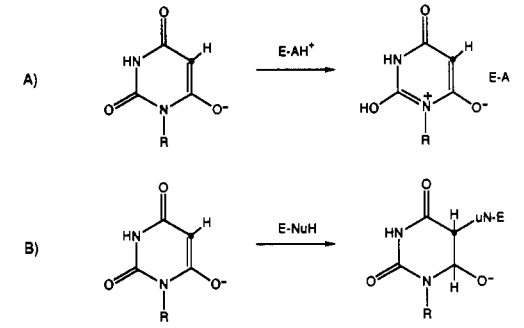
to result from isotopic substitution in OMP (k_H/k_D = 1.00 ± 0.06, n = 10). Table I lists rates (V_i) and rate constants (V_{max}/K_{app}) and their respective ratios determined for OMP and [5-^{2}H]OMP.

Discussion

Beak and Siegel (1976) suggested, on the basis of studies of rates of decarboxylation of model compounds, that conversion of the substrate to a zwitterion at the active site of ODCase could account for 10 of the 12 orders of magnitude in catalytic rate enhancement brought about by the enzyme. Levine et al. (1980) found that BMP was bound extremely tightly by the enzyme and discussed the possibility that the compound might act as a transition-state analogue. These authors considered that three inhibitors of ODCase, namely, UMP, AzaUMP, and BMP, could all form zwitterions at the active site of ODCase but that only the zwitterion of BMP possesses an amide moiety, rendering it most similar in structure to either the zwitterion or ylide intermediate structures in the Beak and Seigel mechanism. These two features could account for the low K_i of 10^{-11} M for BMP in comparison with a K_M value of 10^{-6} M for OMP and K_i values for UMP and AzaUMP of approximately 10^{-7} M.

An alternative mechanism, proposed by Silverman and Groziak (1982), involved addition of an enzymic nucleophile across the 5,6 double bond of the pyrimidine ring system followed by the concerted elimination of CO_2 and the enzymic nucleophile to give product. This mechanism bears a strong resemblance to the mechanism of action of thymidylate synthetase and other enzymes catalyzing electrophilic substitutions on the pyrimidine heterocycle (Santi et al., 1978). Using orotic acid derivatives as models for the enzymatic reaction, Silverman and Groziak showed that the model compounds could undergo addition of a nucleophile to the 5,6 double bond and elimination of CO_2.

Scheme IV: Possible Bound Forms of BMP: (A) Nitrogen Ylide Mechanism and (B) Addition–Elimination Mechanism



If BMP mimics a transition-state or high-energy intermediate for the reaction, then the structure of the bound form of the inhibitor should resemble the structure for the transition state for OMP. Thus if the addition–elimination mechanism were correct, then BMP might be expected to be bound as a covalent adduct, resulting from attack of an enzymic nucleophile at C-5 of the pyrimidine ring. A significant upfield shift of the ^{13}C NMR C-5 resonance would be expected if C-5 was rehybridized from sp^2 to sp^3. The magnitude of this upfield shift, regardless of whether the enzyme nucleophile is sulfur, oxygen, or nitrogen, is expected to be in the neighborhood of 25–50 ppm (Bremser et al., 1982). For the nitrogen ylide mechanism, protonation of the C-2 or C-4 carbonyl oxygen or N-1 to generate the zwitterion intermediate might be expected to result in little or no change in the position of the C-5 resonance. These two alternative modes of binding are depicted in Scheme IV.

When ODCase was titrated with the ^{13}C-enriched BMP, a new resonance at 78.5 ppm was observed. This signal was situated only 0.6 ppm downfield from the resonance of the free inhibitor so that it seems unlikely that covalent addition by an enzymic sulfhydryl or other enzymic nucleophile occurs at C-5 of the inhibitor. Consistent with this conclusion, the UV difference spectrum of the enzyme–inhibitor complex was reported by Levine et al. (1980) to be virtually identical with the spectrum of the inhibitor free in solution, and we have confirmed this observation.

Kinetic effects of substituting hydrogen with deuterium have been used to detect changes in hybridization of reacting centers in substrates of enzyme-catalyzed reactions [for a review, see Kirsch (1977)]. If the rate-limiting transition state for the reaction catalyzed by ODCase involved significant rehybridization of C-5, with sulfur participating as a nucleophile, then a kinetic isotope effect of ~15% might be observed.[2]

For k_{cat}/K_M, two limiting cases may be considered: (1) no isotope effect would be observed if a "nonchemical" event such as substrate binding or an enzyme conformation change determine k_{cat}/K_M,[3] or (2) an isotope effect would be expected if a chemical event caused rehybridization of C-5. If addition or elimination were rate limiting, then the deuterium-labeled substrate might be expected to react more rapidly than the

[2] Bruice and Santi (1982) used this method to detect rehybridization of carbon 6 in the dissociation of the mechanism-based inhibitor 5-fluoro-2′-deoxyuridylate from the ternary complex of inhibitor, methylenetetrahydrofolate, and thymidylate synthetase. Thus, for conversion of the sp^3-hybridized C-6 of 5-fluoro-2′-deoxyuridylate, covalently bound to an enzyme cysteine, to its sp^2 form, free in solution, they found a kinetic effect (k_H/k_T) of 1.23 and a maximal or equilibrium effect (K_H/K_T) of 1.24. Their observed equilibrium and kinetic isotope effects correspond to a deuterium isotope effect of ~1.15 for sp^3 to sp^2.

[3] Since k_{cat}/K_M is ~10^7 M^{-1} s^{-1}, it is possible that diffusion (i.e., substrate binding) may determine k_{cat}/K_M, at least in part.

normal substrate, resulting in an inverse isotope effect ($k_H/k_D < 1.00$).[4] Due to experimental limitations we could not directly measure k_{cat}/K_M but were able to measure the apparent rate constant V_{max}/K_{app} in the presence of the competitive inhibitor AzaUMP. If it is assumed that an isotopic substitution in the substrates should not affect the K_i for AzaUMP, then V_{max}/K_{app} should directly reflect k_{cat}/K_M. No significant isotope effect on V_{max}/K_{app} was observed ($k_H/k_D = 1.00 \pm 0.06$), and it therefore appears that there is no isotope effect for k_{cat}/K_M.

The turnover number, k_{cat}, can be examined in terms of two limiting possibilities.[5] If the addition step of the mechanism were rate limiting, then the deuterium-labeled substrate might be expected to react more rapidly than the normal substrate, giving an inverse isotope effect ($k_H/k_D < 1.00$). If the elimination step were rate limiting and the equilibrium between a noncovalent enzyme–substrate complex and a covalent enzyme–substrate complex strongly favored the covalent complex, then the deuterium-labeled substrate might be expected to react more slowly than the normal substrate, giving a normal substrate isotope effect ($k_H/k_D > 1.00$). At high substrate concentrations, where initial rates are proportional to k_{cat}, we observe no experimentally significant kinetic isotope effect ($V_i^H/V_i^D = 0.99 \pm 0.06$) as a result of substituting deuterium for hydrogen at C-5. This result suggests that neither addition nor elimination of an enzymic nucleophile at C-5 determines k_{cat}.

Spectroscopic properties of enzyme-bound BMP, a possible transition-state analogue inhibitor of ODCase, in addition to the absence of secondary kinetic isotope effects on k_{cat} and k_{cat}/K_M, suggests that carbon 5 of the pyrimidine ring of OMP does not undergo changes in geometry during decarboxylation by ODCase. Therefore, these experiments do not support the existence of covalently bound intermediates on the reaction pathway catalyzed by the enzyme. These results appear, instead, to be consistent with a mechanism (Beak & Siegel, 1976) involving a nitrogen ylide intermediate.

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[4] In theory a preequilibrium isotope effect for the addition step could cancel the isotope effect for the elimination step, resulting in the lack of an observed isotope effect.

[5] These results do not exclude the possibility that k_{cat} might be limited by a process such as product release or a change in enzyme conformation, "masking" a change in geometry at C-5. However, Creasy and Handschumacher (1961) observed a solvent deuterium isotope effect of ~1.5 on k_{cat}, suggesting that a chemical event determines k_{cat}.

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