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Investigation of the Enzymatic Mechanism of Yeast Orotidine-5′-Monophosphate Decarboxylase Using ^{13}C Kinetic Isotope Effects†

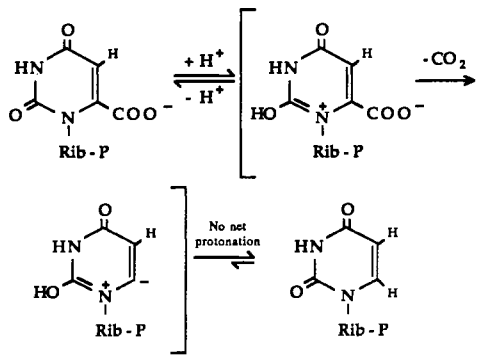
Jeffrey A. Smiley,‡,§ Piotr Paneth,∥,⊥ Marion H. O'Leary,∥ Juliette B. Bell,‡,# and Mary Ellen Jones*,‡
Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7260, and Department of Biochemistry, University of Nebraska—Lincoln, East Campus, Lincoln, Nebraska 68583-0718

Received December 28, 1990; Revised Manuscript Received April 9, 1991

ABSTRACT: Orotidine-5′-monophosphate decarboxylase (ODCase) from *Saccharomyces cerevisiae* displays an observed ^{13}C kinetic isotope effect of 1.0247 ± 0.0008 at 25 °C, pH 6.8. The observed isotope effect is sensitive to changes in the reaction medium, such as pH, temperature, or glycerol content. The value of 1.0494 ± 0.0006 measured at pH 4.0, 25 °C, is not altered significantly by temperature or glycerol, and thus the intrinsic isotope effect for the reaction is apparently being observed under these conditions and decarboxylation is almost entirely rate-determining. These data require a catalytic mechanism with freely reversible binding and one in which a very limited contribution to the overall rate is made by chemical steps preceding decarboxylation; the zwitterion mechanism of Beak and Siegel [Beak, P. & Siegel, B. (1976) *J. Am. Chem. Soc. 98*, 3601-3606], which involves only protonation of the pyrimidine ring, is such a mechanism. With use of an intrinsic isotope effect of 1.05, a partitioning factor of less than unity is calculated for ODCase at pH 6.0, 25 °C. A quantitative kinetic analysis using this result excludes the possibility of an enzymatic mechanism involving covalent attachment of an enzyme nucleophile to C-5 of the pyrimidine ring. The observed isotope effect does not rise to the intrinsic value above pH 8.5; instead, the observed isotope effects at 25 °C plotted against pH yield an asymmetric curve that at high pH plateaus at about 1.035. These data, in conjunction with the pH profile of V_{max}/K_m, fit a kinetic model in which an enzyme proton necessary for catalysis is titrated at high pH, thus providing evidence for the catalytic mechanism of Beak and Siegel (1976).

Orotidine-5′-monophosphate decarboxylase (ODCase,[1] EC 4.1.1.23), catalyzes the conversion of OMP to UMP in the sixth and final step of the de novo pyrimidine biosynthetic pathway. The net reaction involves substitution of the carboxylate moiety at carbon 6 of the pyrimidine ring with a proton, and the reverse reaction has never been observed.

Scheme I: Decarboxylation of OMP by ODCase via Non-covalent, Zwitterion Mechanism[a]



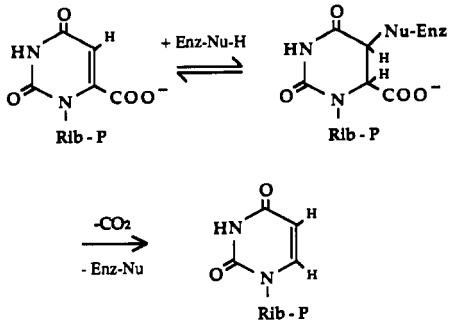
[a] Intermediates in brackets exist at the enzyme active site.

† This work was supported by Grants GM43043 (M.H.O.) and GM 34539 (M.E.J.) from the National Institute of Health. J.B.B. was supported by a National Research Service postdoctoral fellowship NIH 1F32 GM13463 and a Carolina minority postdoctoral scholarship from The University of North Carolina.

* Author to whom correspondence should be addressed.
‡ University of North Carolina.
§ Work done in partial fulfillment of the Ph.D. degree. Present address: Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.
∥ University of Nebraska.
⊥ On leave from Institute of Applied Radiation Chemistry, Technical University of Lodz, Poland.
Present address: Laboratory of Molecular Genetics E3-01, National Institute of Environmental and Health Sciences, Research Triangle Park, NC 27709.

0006-2960/91/0430-6216$02.50/0

Scheme II: Decarboxylation of OMP by ODCase via Nucleophilic Addition Mechanism



ODCase is different from most decarboxylases in that there is no known requirement for a metal ion or cofactor, and the molecular structure of the substrate is unsuitable for a chemical mechanism for decarboxylation similar to that for β-keto acids (Brody & Westheimer, 1979).

Until recently most of the information on the enzymatic mechanism had come from model chemistry or enzyme inhibition studies. On the basis of the observation of rates of non-enzymatic decarboxylation of various OMP analogues and other heterocyclic acids resembling OMP, Beak and Siegel (1976) suggested that the active site of ODCase might function to catalyze the formation of zwitterionic intermediates, followed by decarboxylation to a stabilized nitrogen ylide (Scheme I). Two protons are suggested in this scheme: one that causes the formation of the zwitterion prior to decarboxylation and a second that replaces the carboxylate group at C-6. This model was supported by the observation of Levine et al. (1980) that BMP inhibits the enzyme activity at remarkably low concentration ($K_i \approx 10^{-11}$) and by comparison of the structure of BMP to the transition state of the ODCase reaction proposed by Beak and Siegel (1976). Alternatively, Silverman and Groziak (1982) proposed a reaction involving nucleophilic addition at carbon 5 (Scheme II) after constructing model compounds resembling a proposed enzyme–substrate nucleophilic addition complex that undergo spontaneous decarboxylation at ambient temperatures.

Recently, Acheson et al. (1990) provided evidence against an enzymatic mechanism involving nucleophilic addition at C-5 of OMP with the observation of no significant secondary hydrogen isotope effect [for review, see Kirsch (1977)]. This result would exclude the C-5 nucleophilic mechanism except for the possibility that the enzyme was binding normal or deuterated substrate virtually irreversibly, obscuring the observation of a secondary hydrogen isotope effect. A mechanism involving limited substrate dissociation would require the existence of a large partitioning factor, a mathematical expression composed of a collection of rate constants for individual steps in a multistep enzymatic reaction, which describes the statistical fate of a substrate bound to the active site of an enzyme. Large partitioning factors, indicative of limited substrate dissociation, have been observed for a variety of enzymes, including some decarboxylases [for review, see O'Leary (1991)].

Investigation of ^{13}C isotope effects (O'Leary, 1980) presented an opportunity to address further the issue of the two proposed reaction mechanisms. Determination of ^{13}C isotope effects would lead to estimation of a partitioning factor and thus test the feasibility of the nucleophilic mechanism. For this mechanism to be tenable, the observed ^{13}C isotope effects would necessarily be small, perhaps indistinguishable from unity, due to the virtually irreversible binding implied by the data of Acheson et al. (1990), coupled with the covalent chemical step preceding decarboxylation.

In the zwitterion mechanism, by contrast, only substrate binding and the protonation step (and any enzyme conformation change) would precede the decarboxylation step. If large ^{13}C isotope effects were observable, they would provide direct evidence for noncovalent catalysis such as the nitrogen ylide mechanism. The possibility would then emerge of obtaining additional mechanistic information by measuring isotope effects under altered reaction conditions—most significantly in the case of this mechanism, with altered pH of the reaction medium [cf. O'Leary and Piazza (1981)]. Altered isotope effects under varying conditions might make possible the estimation of the intrinsic isotope effect, the kinetic isotope effect on the carbon–carbon bond-breaking step in the multistep enzyme reaction, and a value that is determined by the transition state of the reaction. In the present study, we measured ^{13}C isotope effects for ODCase under various conditions in order to attempt to determine the intrinsic isotope effect and partitioning factors for this unique enzymatic reaction.

Materials and Methods

Materials. OMP for pH activity curve assays, UMP, 6-aza-UMP, and buffers (MES, MOPS, BTP, CHES, CAPS), were from Sigma Chemical Co. [*carboxyl*-^{14}C]OMP for pH activity curve assays was from Du Pont–New England Nuclear. Reagents and solvents for synthesis of OMP were standard reagent grade.

OMP Synthesis. Synthesis of the substrate for ^{13}C isotope effect assays was carried out on a large scale, since the cost for this reagent in millimolar quantities necessary for these studies was inordinate. OMP was prepared essentially as described (Ueda et al., 1978) with some minor modifications. UMP (disodium salt, 9.5 g, 24 mmol) was converted to 5-bromo-UMP by addition of an equimolar amount of liquid bromine to the UMP suspended in an ice-cold mixture of 90 mL of pyridine and 45 mL of acetic acid. After a 24-h incubation at room temperature with constant stirring, the mixture was analyzed by anion-exchange HPLC (Whatman analytical SAX column, 15-min gradient of 0–0.4 M NH_4HCO_3, 4 mL/min., detection of UV-absorbing material at 260 nm). The reaction was found to be > 99% complete (retention times: for UMP = 6.1 min, for 5-bromo-UMP = 8.5 min). The solvent was removed by evaporation, the residue was washed with repeated addition and evaporation of water and then with dimethylformamide, and the resulting residue was used in the following step without further purification.

The 5-bromo-UMP was dissolved in 250 mL of dimethyl sulfoxide, 2.45 g of (50 mmol) of NaCN was added, and the mixture was stirred at room temperature for 2 days. An additional 613 mg (12.5 mmol) of NaCN, suspended in 100 mL of DMSO, was added, and the mixture was incubated for another 2 days at room temperature. This mixture was analyzed by HPLC, as above, and the extent of the reaction was found to be > 90% (retention time for 6-cyano-UMP = 9.1 min). The solution was diluted with H_2O until all material was dissolved.

KOH (10 M) was added to the crude 6-cyano-UMP solution to give a final concentration of 0.5 M. This mixture was

[1] Abbreviations: ODCase, orotidine-5′-monophosphate decarboxylase; OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; BMP, 1-(5′-phosphoribosyl)barbituric acid; 6-aza-UMP, 6-azauridine 5′-monophosphate; MES, 2-(*N*-morpholino)ethanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfate; BTP, 1,3-bis[[tris(hydroxymethyl)-methyl]amino]propane; CHES, 2-(cyclohexylamino)ethanesulfonate; CAPS, 3-(cyclohexylamino)propanesulfonate.

stirred at room temperature for 1 h, and the initial conversion of 6-cyano-UMP to UMP-6-carboxamide was verified by HPLC analysis (retention time for UMP-6-$CONH_2$ = 7.4 min; major peak > 90%). The solution was then heated in a boiling water bath for 2 h, and the UMP-6-carboxamide was quantitatively converted to OMP (single major peak; retention time = 10.1 min). The apparent increase in purity from about 90% 6-cyano-UMP to a single major peak of OMP as indicated by HPLC analysis is probably attributable to the conversion of small quantities of 6-cyano-UMP to the carboxamide prior to addition of KOH. The solution was acidified to pH < 2 by slurrying with Bio-Rad AG 50W-X8 cation-exchange resin (20–50 mesh, H^+ form), and the product was adsorbed on acid-washed charcoal (Norit A, 2.4 mg/μmol of nucleotide). After being washed with dilute acid, the OMP was eluted from the charcoal with NH_3-saturated 50% ethanol. The eluate was evaporated to dryness and resuspended in a small amount of water, and OMP was precipitated by addition of 150 mL of methanol and 300 mL of acetone. This precipitate was washed with ether and dried under vacuum. The product gave virtually a single peak on HPLC analysis and was quantitated by UV absorbance at 267 nm (ϵ = 9430). A yellow contaminant that persisted throughout the synthesis did not seem to interfere with the enzyme assays.

OMP decarboxylase was prepared from yeast strain 15C carrying the plasmid pGU2, which contains the *ura3* gene encoding ODCase (Lue et al., 1987) as described previously (Acheson et al., 1990), with the same modifications (Bell & Jones, 1991). The enzyme was dialyzed sequentially against multiple change of buffer containing UMP plus phosphate, phosphate alone, and no ligand, to remove 6-aza-UMP used for elution of the enzyme from the affinity columns, as first described by Floyd and Jones (1985).

^{13}C kinetic isotope effects were measured by the competitive method, with samples containing both ^{12}C and ^{13}C, essentially as described by O'Leary (1980). This method measures the isotope effect on V_{max}/K_m. The isotopic ratio of ^{13}C in samples of CO_2 generated by ca. 10% conversion of OMP to products was compared to the ratio in samples of 100% conversion. All reactions contained 10 mM OMP, 0.5 mM EDTA, and 100 mM of one of the following buffers: citrate (pH 4.0), MES (pH 5.4 and 6.0), MOPS (pH 6.8 and 7.4), Tris (pH 8.0), BTP (pH 8.5 and 9.5), CHES (pH 9.5), or CAPS (pH 10.5). For the first set of isotope effect values (pH 6.8, 25 °C), 300 μmol of substrate was used in partial conversion reactions, and 50 μmol of substrate was used in total conversions. These amounts were reduced in subsequent experiments to 150 μmol in partial conversions and 30 μmol in total conversions. For the first set of values, one total conversion sample was analyzed for each partial conversion to obtain k^{12}/k^{13} values; in subsequent experiments, typically one total conversion sample was analyzed for every two partial conversions. Reactions were conducted at room temperature (25 ± 1 °C) or at 0 °C (on ice).

Since contamination of enzyme-generated CO_2 with atmospheric CO_2 can easily interfere in these experiments, a number of precautions common to the study of isotope effects on decarboxylations were taken. Reactions were carried out in vessels equipped with stopcocks and rubber septa to isolate enzyme-generated CO_2 and to allow addition of enzyme as needed. Prior to the addition of enzyme, reaction mixtures were sparged with CO_2-free nitrogen for amounts of time commensurate with pH; high-pH mixtures, expected to contain significant atmospheric CO_2 as bicarbonate, were bubbled more thoroughly. Reaction mixtures containing 20% glycerol were also bubbled extensively, since CO_2 is more soluble in this solution. Collection of CO_2 was attempted occasionally in control experiments without enzyme to ensure that contaminating CO_2 was completely removed. Removal of CO_2 from the enzyme solution was not attempted, since the volume of enzyme solution added was generally less than one-hundredth of the reaction volume, the enzyme was stored at pH 6.0, which would allow very little dissolved bicarbonate, and the maximum possible CO_2 content was calculated to be negligible.

The extent of reaction in scaled-down reaction volumes was monitored spectrophotometrically to provide an estimate of the time needed to achieve 10–20% conversion in the full-scale reactions. This assay utilizes the decrease in absorbance upon conversion of OMP to UMP ($\Delta\epsilon_{285}$ = 2250; Brody & Westheimer, 1979), measured in a 1/200 dilution of the 10 mM OMP reaction mixture using a Milton Roy Spectronic 3000 diode array spectrophotometer. This assay was also used to verify completion of the full conversion reactions. For the isotope effect determinations, reactions were stopped and product CO_2 was purged from the medium by acidification to pH < 2 with phosphoric acid. The CO_2 was collected through use of a high-vacuum distillation apparatus equipped with traps chilled with either liquid nitrogen or dry ice/isopropanol. The amount of the resulting CO_2 and the isotopic content were each measured on a Finnigan Delta S isotope ratio mass spectrometer.

Calculations of ^{13}C kinetic isotope effects were made according to eq 1, where f is the fraction of reaction, R_f is the

$$k^{12}/k^{13} = \log (1 - f)/\log [1 - f(R_f/R_0)] \quad (1)$$

isotopic ratio of the product at the corresponding fraction reaction, and R_0 is the isotopic ratio of the substrate, measured by complete conversion to products (O'Leary, 1980).

Isotope ratios are given as δ ^{13}C, which is calculated from eq 2, where R_{smp} and R_{std} are isotope ratios $^{13}C/^{12}C$ for sample

$$\delta\ ^{13}C = (R_{smp}/R_{std} - 1) \times 10^3 \quad (2)$$

and standard, respectively. The standard for CO_2 is Pee Dee Belemnite.

Assays of ODCase activity to determine the dependence of V_{max}/K_m on pH were done with use of [*carboxyl*-^{14}C]OMP and the $^{14}CO_2$ collection method of Prabhakararao and Jones (1975). Assays were performed at room temperature (25 ± 1 °C) with 25 mM of one of the following buffers at the given pH: citrate (5.0), MES (5.5–6.5), MOPS (7.0–7.5), or Tris (8.0–9.0). Reaction mixtures contained 1.0×10^4 cpm of [*carboxyl*-^{14}C]OMP and various total OMP concentrations from 0.3 to 100 μM. Purified ODCase, at an original concentration of 1.3 mg/mL, as determined by the Bradford protein assay with bovine serum albumin as a standard (Bradford, 1976), was diluted 1/200 in 50 mM phosphate (pH 6.0) plus 20% glycerol, and a maximum of 5 μL of this dilution was used in each 0.5-mL assay. Velocities were calculated as nanomoles per minute per microliter. Assay times were limited to 0.5 min to minimize the error resulting from the observed inactivation of the enzyme upon dilution to a concentration sufficiently low to facilitate these assays. ^{14}C isotope effects (4–10%), which are predictable from the ^{13}C isotope effects, are insignificant compared to the large changes in V_{max}/K_m with varied pH and thus were omitted from the calculation of rates.

Kinetic values were calculated by use of the computer program Enzyme Kinetics (Trinity Software, Campton, NH). These calculated values were then fitted to the equation for

Table I: ^{13}C Kinetic Isotope Effect of ODCase at pH = 6.8, 25 °C[a]

δ ^{13}C	μmol of CO_2[b]	fraction of reaction	k^{12}/k^{13}
−82.25	52.1	0.174	1.0235
−62.66	43.3	1.0	
−84.59	26.8	0.089	1.0248
−62.95	45.4	1.0	
−84.06	34.6	0.115	1.0253
−62.26	40.7	1.0	
−81.57	83.1	0.277	1.0247
−62.36	36.2	1.0	
−84.91	26.7	0.089	1.0254
−62.70	41.5	1.0	

[a] Reaction mixtures were as described under Materials and Methods. [b] Although a total of 50 μmol of CO_2 should be present in total conversion samples, the efficiency of collection is not 100%. This is not a factor in determining the isotopic content, since CO_2 would not be isotopically fractionated by loss of sample during collection. The same lack of efficiency of collection of the partial conversions would not significantly affect the calculation of the isotope effect, since small changes in f for the partial conversions do not significantly alter k_{12}/k_{13} (O'Leary, 1980). [c] k^{12}/k^{13} = 1.0247 ± 0.0008.

log V_{max}/K_m versus pH for two ionizable groups (Segel, 1975) with the computer program SigmaPlot (Jandel Scientific, Corte Madera, CA), from which determination of ionization constants for ODCase was attempted.

Results

The carbon isotope effect on the decarboxylation of OMP by ODCase was measured by analyzing the ^{13}C content of product CO_2 following partial conversion and comparison of the isotopic content to that present in the substrate, as evaluated by analysis of CO_2 following 100% conversion. The isotope effects under the conditions most closely studied (pH 6.8, 25 °C) are listed in Table I. It is apparent from these results that the isotope effect is independent of the fraction of reaction, indicating a lack of contaminating CO_2. No CO_2 could be detected manometrically in samples without enzyme; this criterion was found for other reaction conditions as well. The good agreement among the values for 100% conversion indicates that the enzyme was indeed converting all substrate to product, a condition necessary for reliable calculation of isotope effects. This condition was verified by spectrophotometric analysis of the product, which revealed a decrease in absorbance at 285 nm equal to that predicted by the $\Delta\epsilon_{285}$ for the reaction. In addition, samples that were quenched 5–6 h after enzyme addition gave essentially the same δ ^{13}C values as those that were incubated overnight (data not shown), providing further confirmation of total conversion.

A notable aspect of this data is the abnormally negative δ ^{13}C value of the 100% conversion samples and all partial conversion samples. These low values are indicative of a low amount of ^{13}C present in the substrate and result in a higher potential for error due to contamination but do not affect interpretation of these results. The carboxylate group of the synthesized OMP is derived from sodium cyanide; the cyanide used was found to have substantially higher ^{13}C content than the CO_2 generated enzymatically (δ ^{13}C of NaCN ≈ −29.4 versus δ ^{13}C of OMP ≈ −62.6). Thus, an isotope effect of approximately 1.05 is present in the nucleophilic addition of cyanide to the 5,6 double bond of 5-bromo-UMP.

ODCase displays large ^{13}C kinetic isotope effects that are susceptible to substantial changes upon alteration of the reaction medium. These results are listed in Table II and are summarized graphically in Figure 1. The range of observed isotope effects is unusually large: most of the entire theoretical range of 1.00 to 1.05–1.07 can be observed by alteration of pH, temperature, and/or glycerol content.

Table II: Summary of ^{13}C Kinetic Isotope Effects on the Decarboxylation of OMP by ODCase at Various pH, 25 or 0 °C, in Aqueous Solvent or 20% Glycerol[a]

	k^{12}/k^{13}		
pH	25 °C	0 °C	25 °C in 20% glycerol
4.0	1.0494 ± 0.0006 (4)[b]	1.0506 ± 0.0009 (4)	1.0419 ± 0.0009 (3) [15%][c]
5.4	1.0379 ± 0.0007 (4)	1.0438 ± 0.0006 (3)	ND
6.0	1.0272 ± 0.0004 (4)	ND	ND
6.8	1.0247 ± 0.0008 (5)	1.0367 ± 0.0006 (4)	1.0086 ± 0.0008 (4) [65%]
7.4	1.0221 ± 0.0011 (4)	ND	ND
8.0	1.0319 ± 0.0007 (2)	ND	ND
8.5	1.0372 ± 0.0007 (5)	1.0435 ± 0.0009 (3)	1.0138 ± 0.0003 (2) [63%]
9.5	1.0350 ± 0.0002 (4)	ND	ND
10.5	1.0328 (1)	ND	ND

[a] Content of reaction mixtures is as described under Materials and Methods. [b] Numbers in parentheses indicate number of determinations. [c] Percentages in brackets denote the amount of decrease (toward unity) of the observed isotope effect upon addition of 20% glycerol to the reaction medium. ND, not determined.

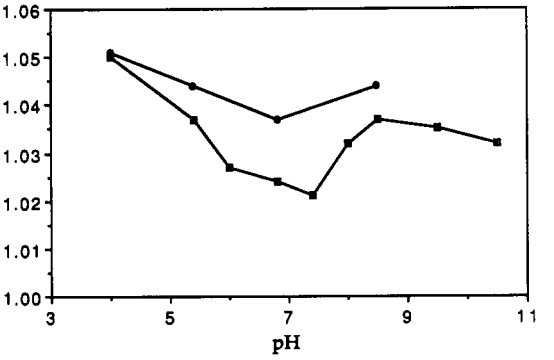


FIGURE 1: Dependence of observed ^{13}C kinetic isotope effects (k^{12}/k^{13}) on pH and temperature. Determination of k^{12}/k^{13} is as described under Materials and Methods at 25 °C (□) and 0 °C (●). Error values are given in Table II.

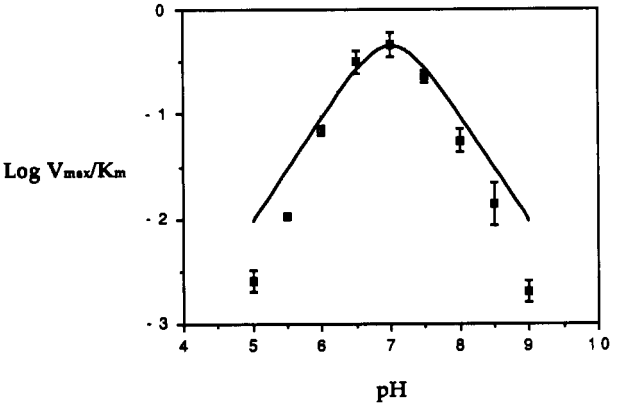


FIGURE 2: Dependence of V_{max}/K_m on pH. Kinetic measurements were made as described under Materials and Methods. Data was fitted by the computer software program SigmaPlot (Jandel Scientific) as described in the Discussion section.

Figure 2 shows the dependence of V_{max}/K_m of ODCase on pH. V_{max}/K_m was determined at various pH values from the inverse of the slopes of Lineweaver–Burk plots of enzyme activity. The possibility that the decline in observed V_{max}/K_m at high pH was due to pH-induced dissociation of active enzyme dimer to inactive monomer was addressed by using varying enzyme concentrations. In assays using increased enzyme concentration, a condition that favors dimer formation (Bell & Jones, 1991), no change from the continued downward

slope of V_{max}/K_m at high pH was observed (data not shown).

The pH optimum of the V_{max}/K_m curve of about 7.0 is in contrast to the optimum velocity at 50 μM OMP of about 5.5 found by Umezu et al. (1971), using acetate and phosphate buffers. Although the pH optimum of V_{max}/K_m could differ from the optimum velocity (approximating V_{max}) as measured by Umezu et al. (1971), we ascribe the difference in pH optima in part to effects on activity by buffers. We have observed a 10–100-fold increase in ODCase activity in assays in the presence of acetate (relative to assays in citrate, tartrate, or MES) near the pK_a's for these buffers. A 4-fold decrease in V_{max}/K_m has been observed in assays containing 50 mM phosphate relative to 50 mM MES (Bell & Jones, 1991). Figure 2 shows large decreases in activity as the pH is altered from the optimum range: a 100-fold decrease in V_{max}/K_m is seen going from pH 7.0 to 5.0, and a similar decrease is seen from pH 7.0 to 9.0.

Discussion

A minimal kinetic model for OMP decarboxylase is as shown in eq 3 where ES represents the Michaelis complex and

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES^{\dagger} \xrightarrow{k_5} EP + CO_2 \qquad (3)$$

$ES^{\dagger}$ represents an enzyme–substrate complex in which the substrate has undergone a chemical step responsible (at least in part) for catalysis. In Scheme I, $ES^{\dagger}$ denotes the enzyme-bound zwitterionic intermediate immediately preceding decarboxylation, while in Scheme II $ES^{\dagger}$ is the covalently bound, 5,6-dihydro intermediate preceding the decarboxylation step. Steps following decarboxylation (k_5) may be kinetically and mechanistically significant, but only those steps including and preceding k_5 are observable in ^{13}C isotope effect studies of decarboxylations. The usual assumptions necessary for facile interpretation of enzyme ^{13}C isotope effects are expected to be valid for OMP decarboxylase: the decarboxylation step is irreversible and is the only step that is sensitive to isotopic substitution at the carboxylate carbon.

From the above notations, the observed ^{13}C isotope effect is defined in eq 4, where k_5^{12}/k_5^{13} is the intrinsic isotope effect

$$k^{12}/k^{13} = \frac{k_5^{12}/k_5^{13} + (k_5/k_4)(1 + k_3/k_2)}{1 + (k_5/k_4)(1 + k_3/k_2)} \qquad (4)$$

and $(k_5/k_4)(1 + k_3/k_2)$ is the partitioning factor. With use of abbrevations—Obs for the observed isotope effect, Int for the intrinsic isotope effect, and R for the partitioning factor—eq 4 can be rearranged to give

$$R = [(\text{Int} - 1)/(\text{Obs} - 1)] - 1 \qquad (5)$$

and thus the partitioning factor can be expressed simply in terms of the ratio of the differences of the intrinsic and observed isotope effects from unity.

Pattern of Kinetic Isotope Effects at pH ≤ 6.8: Observation of Intrinsic Isotope Effect. The value for the observed ^{13}C isotope effect at pH 6.8, 25 °C, of 1.0247 ± 0.0008 is representative of a multistep reaction in which decarboxylation is not entirely rate-limiting. The observed value rises to 1.0367 ± 0.0006 upon a lowering of the temperature to 0 °C (Table II). The influence of temperature on isotope effects of enzyme reactions can involve only very modest changes in the intrinsic isotope effect; in the temperature range available for enzyme assays, altered isotope effects are nearly entirely attributable to variations in the partitioning factor (O'Leary, 1978). The change in partitioning factor is probably the result of decreased rates of chemical reactions (k_3, k_4, and k_5) and not substrate dissociation (k_2), leading to changes in the ratios k_5/k_4 and k_3/k_2.

Lowering the pH of the reaction also results in a significant change in the observed isotope effect; the value increases to 1.0379 ± 0.0007 at pH 5.4, 25 °C (Table II). Again, the influence of pH on the observed isotope effect is presumed to be a variation of the partitioning factor and not of the intrinsic isotope effect (O'Leary, 1978), since the H^+ concentration would not be expected to change the structure of the transition state. In the case of altered pH, the change in partitioning factor can result from a change in dissociation (k_2) and possibly the pre-decarboxylation chemistry (k_3 and k_4), depending on the nature of the step but probably not on the decarboxylation step itself (k_5). The effects of lowering both temperature and pH were found to be cumulative as the observed isotope effect at pH 5.4, 0 °C is 1.0438 ± 0.0006 (Table II).

When the pH was lowered further to 4.0 (25 °C), the observed value rose to 1.0494 ± 0.0006 (Table II). This value approaches the range of 1.05–1.07 for intrinsic isotope effects predicted for decarboxylation reactions (Marlier & O'Leary, 1986). Since intrinsic isotope effects for decarboxylations of aromatic carboxylic acids are expected to be lower than those for aliphatic carboxylates (Dunn, 1977), we expected that the intrinsic isotope effect for decarboxylation of OMP might be at the low end of this range, and we predicted that the value for the enzymatic reaction observed at pH 4.0 might be close to the intrinsic value and could not be significantly raised further by lowering temperature. This prediction was verified by the observation of an isotope effect of 1.0506 ± 0.0009 at pH 4.0, 0 °C (Table II). Thus we conclude that ODCase catalyzes the decarboxylation of OMP with an intrinsic isotope effect of about 1.05 and that, under extreme medium conditions (pH 4.0, 0 °C), the full intrinsic effect is observed and the decarboxylation step is fully rate-determining.

Effect of Glycerol on ^{13}C Kinetic Isotope Effects. The decrease in the observed ^{13}C isotope effect at pH 6.8, 25 °C, by the addition of 20% glycerol is substantial: the value falls to 1.0086 ± 0.0008 (Table II). The influence of decreasing medium polarity on isotope effects of decarboxylations has been observed previously for chemical (Marlier & O'Leary, 1986) and enzymatic (O'Leary & Piazza, 1981) reactions, and a number of factors can contribute to this influence. Again, we do not predict that the decrease in the observed isotope effect results from a decreased intrinsic isotope effect but instead from a change in the partitioning factor, in which case the decarboxylation step has become less rate-determining.

The assertion that the intrinsic isotope effect is being observed at pH 4.0 can be qualitatively assessed by addition of glycerol to the reaction medium. If the observed isotope effect at pH 4.0 is indeed very near the intrinsic value, then addition of glycerol should have little influence on the partitioning factor, which is near zero, and thus little influence on the observed isotope effect. By contrast, at pH 6.8 or 8.5, where the partitioning factors are near unity, we would expect more of a decrease in the observed isotope effect. This is, in fact, what is seen: at the higher pH levels, the isotope effect is reduced by about 63%, whereas at pH 4.0 the decrease is only about 15% (Table II). Thus, the influence of glycerol on the observed isotope effects reinforces our conclusion that the intrinsic isotope effect is about 1.05 and is observed in aqueous solvent at pH 4.0.

For an increase in the partitioning factor to occur, one or more of the individual rate constants must be altered in such a way as to increase k_5/k_4 or k_3/k_2 or both. The rate constant for which the alteration by decreasing medium polarity is best

understood is k_5, the decarboxylation step. As has been discussed previously (Marlier & O'Leary, 1986; O'Leary & Piazza, 1981, and references therein), the rates of chemical decarboxylations are enhanced by decreasing the polarity of the reaction medium. The enhancement of the decarboxylation step by decreasing medium polarity probably results from the chemical nature of the reactants and products: neutralization of a charged carboxylate moiety to an uncharged CO_2 molecule, a reaction favored by a nonpolar medium. In a multistep enzyme reaction, the decarboxylation step would therefore be less rate-determining; the decrease in the observed isotope effect could be explained in part by this factor.

Presumably, the addition of glycerol to the reaction medium would change the polarity of the active site, if the medium effect on the decarboxylation step is realized in the enzymatic reaction. If the ODCase reaction involves a zwitterionic intermediate, as proposed by Beak and Siegel, the neutralization of the transient positive charge on the pyrimidine ring during catalysis would also contribute to enhancement of the decarboxylation step upon decreasing medium polarity. Neutralization of zwitterionic intermediates has been discussed for the case of arginine decarboxylase, which exhibits decreased ^{13}C isotope effects in less polar environments (O'Leary & Piazza, 1981), and for the chemical decarboxylation of a thiamine pyruvate analogue (Crosby et al., 1970). The role of ODCase may also include the positioning of the carboxylate group into a hydrophobic space to effect decarboxylation, as has been observed in the crystal structure of histidine decarboxylase (Gallagher et al., 1989). The enhancement of the decarboxylation step by addition of glycerol to the reaction medium would be consistent with this active site structure.

The addition of glycerol to the ODCase reaction medium may also have the effect of decreasing substrate dissociation (k_2). The substrate OMP is almost certainly highly solvated when in free aqueous solution, and the binding of OMP to the active site of ODCase most likely involves desolvation of the substrate, as suggested previously by Levine et al. (1980). Conversely, dissociation of OMP from the active site involves the thermodynamic advantage of resolvation; in 20% glycerol, the hydrogen-bonding capacity of the medium is reduced, and thus the thermodynamic advantage of dissociation would be lessened. Dissociation of OMP from ODCase in glycerol would thus be expected to be slower, and the decreased observed isotope effect may be partially explained in this way.

Analysis of Kinetic Data for C-5 Nucleophilic Mechanism. The observation of large ^{13}C isotope effects for ODCase, values that approach the predicted intrinsic isotope effect, suggests that no covalent chemical step is occurring prior to decarboxylation. Only an unusually fast covalent step could allow the decarboxylation step to be fully rate-determining, which appears to be the case at pH 4.0. A survey of decarboxylases (O'Leary, 1988) shows that amino acid decarboxylases, involving covalent attachment of either a pyridoxal or pyruvate cofactor, display observed isotope effects that are substantially less than the predicted intrinsic isotope effects.[2] By contrast, formate dehydrogenase, a single-step, CO_2-generating enzyme, displays a full intrinsic isotope effect (Blanchard & Cleland, 1980).

On the basis of the data of Acheson et al. (1990), we can evaluate the C-5 nucleophilic mechanism from a quantitative perspective. If the C-5 nucleophilic mechanism is operational, then the following equation for the observed secondary deuterium isotope effect on V_{max}/K_m, with use of the rate constants defined in eq 3 and the designation of $ES^{\dagger}$ as a covalent sp^3 intermediate bound at C-5, must be solvable:

$$k^H/k^D = \frac{K_H/K_D + (k_5/k_4)(k_3^H/k_3^D + k_3/k_2)}{1 + [(k_5/k_4)(1 + k_3/k_2)]} \quad (6)$$

where K_H/K_D is the equilibrium isotope effect on the formation of the covalent $ES^{\dagger}$ complex at C-5 of the substrate, k_3^H/k_3^D is the intrinsic isotope effect on k_3, the deuterium-sensitive step of the proposed reaction, and the bracketed collection of rate constants in the denominator is the partitioning factor. Acheson et al. (1990) measured k^H/k^D for ODCase to be 1.00 ± 0.06.[3] The partitioning factor is calculated from our data to be 0.84 from eq 5, with the observed ^{13}C isotope effect of 1.0272 ± 0.0004 at pH 6.0 (Table II) and an estimated intrinsic ^{13}C isotope effect of 1.05.

The equilibrium isotope effect (K_H/K_D) can be estimated from data of Bruice and Santi (1982), who studied the dissociation of the nucleophilic addition complex of thymidylate synthase with 5-fluoro-2′-deoxy-UMP, a reaction that resembles the reverse reaction of covalent attachment of a nucleophile of ODCase to C-5 of OMP. Their value of K_H/K_T = 1.24 corresponds to $K_H/K_D \approx 1.15$ and a $K_H/K_D \approx 0.87$ for a similar reaction in the reverse direction, such as the one postulated for ODCase catalysis. K_H/K_D for a reaction of this type is expected to be in the range of 0.8–0.9 (Lowry & Richardson, 1981), so this estimated value seems quite reasonable. Inserting this estimated value and the values for k^H/k^D and the partitioning factor into eq 6 yields

$$1.00 = \frac{0.87 + (k_5/k_4)(k_3^H/k_3^D + k_3/k_2)}{1.84} \quad (7)$$

Following rearrangement of eq 7, the equation for the value of the partitioning factor, $0.84 = (k_5/k_4)(1 + k_3/k_2)$, can be used to yield

$$1.15 = \frac{k_3^H/k_3^D + k_3/k_2}{1 + k_3/k_2} \quad (8)$$

and solving for k_3^H/k_3^D for an sp^2 to sp^3 conversion, which must necessarily be less than unity due to theoretical considerations (Kirsch, 1977), is impossible, since k_3/k_2 in eq 8 cannot be less than zero.

For eq 8 to be solvable, the values inserted into eq 6 must be stretched beyond the limits of error and reason. Unless the observed k^H/k^D is outside the range of error reported by Acheson et al. (1990), the estimated K_H/K_D must be inaccurate and much closer to unity, in conflict with theoretical expectations. Recalculation of the partitioning factor using eq 5 and an intrinsic ^{13}C isotope effect of 1.06 would not affect the above calculations substantially. These considerations—coupled with the observation of even larger ^{13}C isotope effects and smaller partitioning factors at lower pH, which would require a smaller observed k^H/k^D and a more unlikely $K_H/$

[2] An exception to this general rule is found with alternate substrates of arginine decarboxylase, homoarginine, and norarginine (O'Leary & Piazza, 1981). With these substrates it is observed that the overall rate is decreased by a factor of 100–1000, and since the K_m values are approximately equal, it is the decreased rate of decarboxylation that is believed to contribute entirely to the decreased overall rate, and thus the isotope effect observed is near the intrinsic value.

[3] The actual isotope effect measured by Acheson et al. (1990) is an isotope effect on an apparent rate constant, V_{max}/K_{app}. Due to experimental limitations, i.e., K_m at pH 6.0 is approximately 1 μM and thus too low for spectrophotometric assays, these assays for determination of apparent k^H/k^D were performed in the presence of 260 μM 6-aza-UMP, a competitive inhibitor.

K_D—lead us to dismiss the C-5 nucleophilic mechanism of catalysis by ODCase.

Kinetic Isotope Effects at High pH. The observed ^{13}C isotope effect reaches an apparent minimum at pH 7.4 and rises sharply to higher values at pH 8.0 and 8.5 (Figure 1). These points initially indicated that the observed isotope effect might rise to the same maximum observed at low pH, resulting in a somewhat symmetrical plot over the pH range studied. However, the observed isotope effect at high pH reached a plateau at approximately 1.035, a value distinctly lower than the highest value observed at low pH.

The unlikely possibility that the intrinsic isotope effect is lower at high pH, and that the enzyme catalyzes decarboxylation at this lowered intrinsic value, is dismissed from consideration by the observation of a higher observed isotope effect at lower temperature. At 0 °C, the value at pH 8.5 is 1.0440 ± 0.0009 (Table II), clearly higher than the value at 25 °C. The possibility that the change in the pattern of isotope effects was due to use of the more hydrophobic buffers CHES and CAPS was ruled out by the measurement of virtually the same isotope effect at pH 9.5 in BTP or CHES buffers (data not shown).

The observed isotope effects over the pH range studied, in combination with the pH dependence of V_{max}/K_m (Figure 2), suggest a kinetic scheme and mechanistic aspects that are in accord with a chemical route for decarboxylation of OMP to UMP involving protonation of the substrate by the enzyme, similar to that proposed by Beak and Siegel (1976). Due to the sensitivities of V_{max}/K_m and the observed isotope effect to changing pH, the kinetic scheme in eq 3 must be modified to include protonated and deprotonated forms of the enzyme. Construction of this modified kinetic scheme begins with consideration of the isotope effect data at low pH, where the observed isotope effect rises to very near the intrinsic isotope effect. This observation can be explained by the following modification of eq 3:

$$\begin{array}{ccccccc} HE & + S & \rightleftharpoons & HES & \rightleftharpoons & HES^{\dagger} & \\ \uparrow\downarrow & & & \uparrow\downarrow & & \uparrow\downarrow & \\ E & + S & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & ES & \underset{k_4}{\overset{k_3}{\rightleftharpoons}} & ES^{\dagger} & \xrightarrow{k_5} EP + CO_2 \end{array} \quad (9)$$

where HE and HES represent protonated forms of free enzyme and enzyme–substrate complex, respectively. This partial scheme predicts that, at decreasing pH, a larger percentage of the total enzyme is protonated to an inactive form, capable of binding substrate but incapable of catalysis. At low pH, nearly all of the enzyme–substrate complex would be present as HES or $HES^{\dagger}$, conversion of $ES^{\dagger}$ to products would be nearly entirely rate-determining (k_5 would approach zero), and the isotope effect would approach the intrinsic value; the isotope effect data and the dependence of V_{max}/K_m on pH reported herein fit this model. If the $HES^{\dagger}$ complex were capable of catalysis to a significant degree, the isotope effect would not approach the intrinsic value and V_{max}/K_m would not continue downward at low pH.

Consideration of the kinetic data at high pH leads to further modification of eq 9. The continued downward slope of V_{max}/K_m values at high pH (Figure 2) indicates that deprotonated forms of the enzyme must exist, and that these deprotonated forms are unable to catalyze decarboxylation, yet the plateau of isotope effect values (Figure 1) suggests that the pattern of deprotonated enzyme species does not parallel the corresponding protonated forms. The following scheme agrees well with the presented kinetic data and supports the chemical mechanism for catalysis of Beak and Siegel (1976).

$$\begin{array}{ccccccc} HE & + S & \rightleftharpoons & HES & \rightleftharpoons & HES^{\dagger} & \\ \uparrow\downarrow & & & \uparrow\downarrow & & \uparrow\downarrow & \\ E & + S & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & ES & \underset{k_4}{\overset{k_3}{\rightleftharpoons}} & ES^{\dagger} & \xrightarrow{k_5} EP + CO_2 \\ \uparrow\downarrow & & & \uparrow\downarrow & & & \\ E^{-} & + S & \rightleftharpoons & E^{-}S & & & \end{array} \quad (10)$$

E^- and E^-S represent deprotonated forms of free enzyme and enzyme–substrate complex, respectively. As the pH is continually raised, more of the enzyme is present as inactive, deprotonated forms, which results in a continued downward slope of V_{max}/K_m, in agreement with Figure 2. The above scheme also agrees with the observed plateau of the isotope effect at high pH. The scheme predicts that, at high pH, the enzyme is present predominantly as deprotonated forms, but k_3, not k_5, would become rate-determining. Thus, k_3 would approach zero; the partitioning factor (eq 4) would not approach zero but instead would approach k_5/k_4, and the observed isotope effect would not reach the intrinsic value.

The catalytic mechanistic assumption that follows from this scheme is that ES represents an enzyme–substrate complex that is poised to protonate the substrate, leading to $ES^{\dagger}$, in the zwitterion mechanism of Beak and Siegel. As pH increases, the catalytic proton in ES is titrated and ES becomes E^-S. Thus, E^-S cannot proceed to a deprotonated, activated enzyme–substrate complex ($E^-S^{\dagger}$) because the catalytic proton that produces the activated complex is no longer present; thus $E^-S^{\dagger}$ does not exist. If $E^-S^{\dagger}$ did exist, then either the V_{max}/K_m values at high pH (Figure 2) would reach a minimum plateau or the isotope effect (Figure 1) would continue upward to the intrinsic value.

For the mechanistic scheme illustrated by eq 10 to be consistent with the isotope effect data, k_3/k_2 must not be close to zero at neutral pH, i.e., the chemical step leading to $ES^{\dagger}$ must proceed at a rate that is significant compared to substrate dissociation. If k_3 is a protonation step, as suggested in the zwitterion mechanism of Beak and Siegel, it would be expected to be fast and proceed at a rate competitive with substrate dissociation. Additionally, the proton on the chemical group of ES that is titrated to yield E^-S must be directly transferred to the substrate and no longer accessible to solvent in $ES^{\dagger}$. This condition is in agreement with the observation that the isotope effect, determined predominantly by k_5/k_4 at high pH, is relatively unaffected above pH 8.5 and the deduction that an activated, deprotonated form of ES does not exist.

The shape of the curve in Figure 2 is indicative of two titratable chemical groups on the free enzyme or substrate, one of which must be protonated and the other deprotonated, with similar ionization constants. Furthermore, the lack of a plateau at optimal pH in Figure 2 is likely the result an acidic pK—i.e., an ionization constant of a chemical group that must be deprotonated for activity—which is higher than the accompanying basic pK. The pH optimum indicates that the average of these two values is about 7.0. Figure 2 is fitted to a curve for pH dependence of V_{max}/K_m for a diprotic system (Segel, 1975) with an acidic pK of 7.5 and a basic pK of 6.5; raising the first value and lowering the second does not change the shape of the curve appreciably, and thus it is not possible to determine with certainty the ionization constants from the data in Figure 2 alone. The steeper decline in V_{max}/K_m in Figure 2 from pH 8.5 to 9.0, approaching slope = −2, is indicative of another deprotonation. The existence of doubly ionized forms of the enzyme (e.g., E^{-2} and $E^{-2}S$ in eq 10) would not necessitate change in the above interpretation of the presented data.

From the above discussion, it appears that the titratable group that must be protonated for enzyme activity is an enzyme functional group responsible for protonating the substrate in the zwitterion mechanism. This catalytic functional group could be positively charged in the active form of the enzyme and titrated to become neutral, or neutral in the active form and titrated to become negatively charged. Studies of enzyme inhibition showed that ligands with ionizable pyrimidine rings bind most tightly to the enzyme as anions (Levine et al., 1980; Handschumacher, 1960), indicating the presence of a positively charged group at the active site. From this we could predict that this positively charged active site group is the catalytic functional group and is titrated to become neutral. The titratable group that must be deprotonated for activity may be an enzyme carboxylate group, possibly situated within the binding site for the substrate carboxylate, similar to the active site of histidine decarboxylase (Gallagher et al., 1989). Alternatively, this titratable group may be the phosphate of the substrate.

In conclusion, we have shown that ODCase displays large ^{13}C isotope effects that can be altered by changing either the pH, temperature, or glycerol content of the reaction medium. The data preclude the possibility of a nucleophilic addition at C-5 of the substrate and indicate that k_3, the chemical step leading to catalysis, is a protonation step in a catalytic mechanism similar to that proposed by Beak and Siegel (1976). Resolution of the structure of ODCase crystals (Bell et al., 1991) may indicate the enzyme functional groups directly involved in catalysis, and further studies to gain more experimental evidence for the chemical mechanism for catalysis are ongoing.

ACKNOWLEDGMENTS

We thank R. V. Wolfenden and W. W. Cleland for helpful discussions during the preparation of the manuscript.

Registry No. ODCase, 9024-62-8; OMP, 2149-82-8; ^{13}C, 14762-74-4; glycerol, 56-81-5.

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