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Determination of the Mechanism of Orotidine 5′-Monophosphate Decarboxylase by Isotope Effects†

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ABSTRACT: Orotidine 5′-monophosphate shows a ^{15}N isotope effect of 1.0036 at N-1 for decarboxylation catalyzed by orotidine 5′-monophosphate decarboxylase. Picolinic acid shows a ^{15}N isotope effect of 0.9955 for decarboxylation in ethylene glycol at 190 °C, while *N*-methyl picolinic acid shows a ^{15}N isotope effect of 1.0053 at 120 °C. The transition state for enzymatic decarboxylation of orotidine 5′-monophosphate resembles the transition state for *N*-methyl picolinic acid in that no bond order changes take place at N-1. This rules out enolization to give a quaternary nitrogen at N-1 in the enzymatic mechanism and suggests a carbanion intermediate stabilized by simple electrostatic interaction with Lys-93. The driving force for the reaction appears to be ground-state destabilization resulting from charge repulsion between the carboxyl of the substrate and Asp-91.

Orotidine 5′-monophosphate decarboxylase (ODCase)[1] catalyzes the decarboxylation of OMP to give UMP (Figure 1). This enzyme is of interest for two reasons. The catalytic proficiency is one of the highest known for any enzyme (*1*). However, the mechanism by which the enzyme achieves this tremendous catalytic effect has been unknown. Also, ODCase catalyzes the critical final step in the pyrimidine biosynthetic pathway (*2*).

Several mechanisms have been proposed for the enzymatic decarboxylation of OMP based on nonenzymatic reactions with model compounds. Silverman and Groziak studied models of a covalent adduct of OMP and proposed that ODCase might also form a covalent adduct (*3*). In contrast, model studies by Beak and Siegel suggested the possibility of catalysis involving formation of an ylide with a positive charge at N-1 (*4*). This mechanism might additionally involve formation of a low barrier hydrogen bond with the ylide intermediate (*5*). Lee and Houk calculated that stabilization of a carbanion intermediate could involve a carbene resonance structure without a positive charge at N-1 (*6*). Studies with model compounds were consistent with both ylide and carbene stabilization of a carbanion intermediate (*7*). Very recently, the crystal structure of ODCase has been solved with three different inhibitors bound in the active site (*8*−*10*).

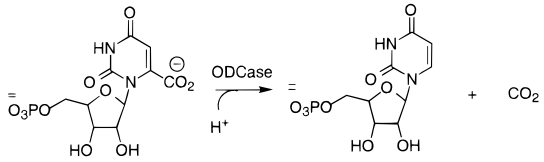

FIGURE 1: Decarboxylation of OMP by ODCase.

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[1] Abbreviations: ODCase, orotidine 5′-monophosphate decarboxylase; OMP, orotidine 5′-monophosphate; UMP, uridine 5′-monophosphate; BMP, barbituric acid 5′-monophosphate. Residue numbers throughout refer to the yeast enzyme.

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Several prior observations argued against the covalent mechanism (*11*, *12*). The remaining mechanisms differ in the bonding around N-1 prior to decarboxylation. We have therefore determined the kinetic ^{15}N isotope effects at N-1 for the enzymatic decarboxylation of OMP. The kinetic ^{15}N and ^{13}C isotope effects for nonenzymatic decarboxylation of two model compounds were also determined for comparison.

EXPERIMENTAL PROCEDURES

Materials. Picolinic acid, trimethyloxoniumtetrafluoroborate, bromine, ethylene glycol, 5% rhodium-on-alumina, 1,2 dichloroethane, and Nessler's Reagent were from Aldrich. Quinoline from Aldrich was vacuum distilled from sodium and stored in a desiccator with P_2O_5. Uridine-5′-monophosphate, OMP decarboxylase, and alkaline phosphatase were from Sigma. Sodium cyanide from J. T. Baker was recrystallized from ethanol−water. Mercuric oxide was from Mallinckrodt. Ethyl picolinate was from Lancaster. Sodium hypobromite was prepared by slow addition of bromine to cold 17 N NaOH. This solution was allowed to stand at 4 °C for 5 days, and the precipitate was removed by filtration.

N-Methyl Picolinic Acid. Trimethyloxoniumtetrafluoroborate (10 g) and ethyl picolinate (5 mL) were dissolved in 100 mL of 1,2-dichloroethane, and the solution was heated to reflux with stirring for 3 h. The solution was cooled to 0 °C overnight, and the precipitate was removed by filtration. The solvent was removed by evaporation in vacuo, and the orange liquid was taken up in 100 mL of water. The solution was loaded onto a Dowex AG1-X8 anion-exchange column (2.5 cm × 20 cm) in the hydroxide form and the column was washed with water. The eluate was monitored at 272 nm, and fractions containing product were pooled and made acidic with 17 mL of 2 N HCl. The water was removed by evaporation in vacuo, and the dried *N*-methyl picolinate hydrochloride was stored in a desiccator over P_2O_5.

Orotidine 5′-Monophosphate. Disodium uridine 5′-monophosphate (4 g) was taken up in 40 mL of pyridine and 20 mL of acetic acid. The solution was chilled to 4 °C, and bromine (0.52 mL) was added with stirring. The ice bath was removed, and the solution was stirred for 16 h. The solution was evaporated to an orange foam which was repeatedly taken up in 100 mL of water and evaporated to dryness. The orange oil was taken up in 50 mL of DMF and evaporated to dryness. The oil was dissolved in 100 mL of DMSO and sodium cyanide (1 g) was added with stirring. After 2 days, sodium cyanide (0.25 g) suspended in 40 mL of DMSO was added, and stirring continued for 2 days. Water was added to the solution until all precipitated material dissolved (about 100 mL). The solution was made basic with 13 mL of 10 N KOH, and the solution was stirred at room temperature for 1 h, then in a boiling water bath for 2 h. The solution was cooled to room temperature and Dowex 50W−X2 cation-exchange resin in the proton form was added until the pH was less than 2.

The cation-exchange resin was removed by filtration, and the solution was loaded onto an acid-washed charcoal column (2.5 × 35 cm), 20−40 mesh. The column was washed with 1 L of 0.1 N HCl and eluted with 50% ethanol saturated with ammonia. The eluate was monitored at 267 nm, and fractions containing UV absorbance were pooled and evaporated to dryness. The clear oil was taken up in 100 mL of water and loaded onto a Dowex AG1-X8 anion-exchange column (3.5 × 45 cm) which was washed with 1 L of water. The column was eluted with 0.01 N HCl/0.25 N NaCl and the eluate was monitored at 267 nm. Several UV-absorbing peaks were observed, and all were collected separately and evaporated to dryness. Orotidine 5′-monophosphate was located by UV−vis spectroscopy and ^1H NMR spectroscopy, and the OMP was dissolved in a small volume of water. This solution was loaded onto an acid-washed charcoal column (2.5 × 35 cm), 20−40 mesh. The column was washed with 1 L of water and eluted with 50% ethanol saturated with ammonia. The eluate was monitored at 267 nm, and fractions containing OMP were pooled and evaporated to about 20 mL. To this solution was added 100 mL of methanol and 200 mL of acetone. The solution immediately became cloudy with a white precipitate. The solution was placed at 4 °C overnight. The precipitate was collected by filtration and stored in a desiccator with P_2O_5.

Heavy Atom Isotope Effects on Decarboxylation of Picolinic Acid. Picolinic acid (12.3 mg) was placed in a dry quartz tube (24 cm × 9 mm o.d., 7 mm i.d.), and the tube was fitted with a septum. Anhydrous ethylene glycol (5 mL) was added by cannula, and the septum was replaced with a Cajon fitting connected to an adapter with a ground glass joint. The apparatus was placed on a vacuum line equipped with a nitrogen gas reservoir. The quartz tube was evacuated and back-filled with dry CO_2-free nitrogen gas. A small oven was raised to surround the portion of the quartz tube containing the ethylene glycol solution, and the oven was heated to 190 °C. The samples were heated from 15 to 75 min for partial decarboxylation reactions or 8 h for total decarboxylation. The oven was then removed, and the CO_2 produced during the reaction was distilled through dry ice−2-propanol traps on a high vacuum line and collected in a sample chamber using liquid nitrogen. The gas was quantified with a standardized mercury manometer and analyzed by IRMS.

The ethylene glycol solution was diluted to 80 mL with water and titrated to pH 10 with KOH. The sample was loaded onto a Dowex AG1-X8 column (2.5 cm × 10 cm) in the hydroxide form. The column was washed with 500 mL of water and eluted with 0.1 N HCl. Picolinic acid eluted as soon as all of the resin had converted to the chloride form and was located by UV assay. Fractions containing picolinic acid were evaporated to dryness. The amount of picolinic acid present was determined by UV assay at 265 nm and comparison to a standard curve. The sample was transferred to a quartz tube which was filled with CuO (10 g), Cu (0.2 g), and silver (0.2 g). The tube was evacuated, sealed with a torch, and heated to 850 °C for 2 h. The tube was cracked on a high vacuum line and the nitrogen gas produced was distilled through dry ice-2-propanol and liquid nitrogen traps and collected on molecular sieves chilled with liquid nitrogen. The purified nitrogen was analyzed by IRMS. Samples of unreacted picolinic acid were sealed in quartz tubes and analyzed as above to determine the abundance of ^{15}N in the starting material.

Heavy Atom Isotope Effects on Decarboxylation of N-Methyl Picolinic Acid. The conditions used for decarboxylation of picolinic acid were also used with *N*-methyl picolinic acid with two exceptions. Quinoline (40 μL) was added by

syringe to the solution prior to evacuation, and the reactions were only heated to 120 °C for 20−45 min for partial decarboxylation. Total decarboxylation reactions were heated to 120 °C for 4 h.

The ethylene glycol solutions were diluted to 25 mL with water and titrated to pH 10.5 with KOH. The solutions were extracted three times with an equal volume of ethyl acetate to remove the quinoline. The solutions were titrated to pH 2.5 with HCl and loaded onto a Dowex 50W-X2 cation-exchange column (2.5 × 20 cm) in the proton form. The column was eluted with 500 mL of 0.01 N HCl followed by 1 N HCl. *N*-Methyl picolinic acid eluted with dilute HCl and was located by UV assay at 272 nm. *N*-Methyl pyridine eluted with concentrated HCl and was located by UV assay at 258 nm. Fractions containing these compounds were evaporated to dryness. The amount of *N*-methyl picolinic acid present was determined by assay at 272 nm and comparison to a standard curve. The samples were transferred to quartz tubes, and the abundance of ^{15}N in each sample was determined as described above.

^{15}N Isotope Effects with Orotidylate Decarboxylase. Orotidine 5′-monophosphate (40 mg) was dissolved in 10 mL of 20 mM Mes/5 mM DTT. The solutions were titrated to pH 6.5 and 0.2 mg of orotidylate decarboxylase was added. The solutions were stirred for 3−5 days. The reactions were diluted to 50 mL with water and loaded onto a Dowex AG1-X8 anion-exchange column (2.5 × 25 cm) in the chloride form. The column was eluted with 0.1 N HCl until UMP stopped eluting, as determined by UV assay at 262 nm. The column was then eluted with 1 N HCl until OMP stopped eluting as determined by UV assay at 267 nm. Fractions containing nucleotides were pooled and evaporated to dryness. The nucleotides were taken up in 10 mL of 20 mM Tris and titrated to pH 8.0 with KOH and the concentration of each nucleotide was determined by UV assay. Alkaline phosphatase (9 units) was added and the reactions were stirred overnight.

The samples were diluted to 50 mL and loaded onto a Dowex AG1-X8 column in the hydroxide form. The columns were washed with 250 mL of water and eluted with 0.1 N HCl until nucleosides stopped eluting. The nucleoside solutions were repeatedly evaporated to dryness and taken up in a small volume of water and then titrated to pH 6.5. The solutions were evaporated to about 0.5 mL. The samples were injected onto a C18 HPLC column and eluted with 5% methanol. Eluate containing the desalted nucleosides was evaporated to dryness, and the nucleosides were taken up in 10 mL of water. To each solution was added 30 mg of 5% rhodium-on-alumina, and the samples underwent hydrogenation at 30 psi for 16 h. The catalyst was removed by filtration, and the solvent was removed by evaporation. Dihydrouridine was taken up in 5 mL of 6 N HCl while dihydroorotidine was taken up in 5 mL of 4 N HCl. The samples were heated to 90 °C for 3 days in Parr acid digestion bombs. The acidic samples were evaporated to dryness, and the soot remnant was taken up in 10 mL of water. The ammonium content was determined by Nessler's assay at 425 nm. The samples were titrated to pH 11 with KOH and evaporated to remove ammonia from the N-3 of the bases. The remaining soot was dissolved in a small volume of water, and the samples were transferred to long-neck Kjeldahl flasks.

To each Kjeldahl flask were added 1.5 mL of 0.46 N HgO in 4.32 N sulfuric acid, 1.5 g of potassium sulfate, and 3 mL of concentrated sulfuric acid. The solutions were heated to reflux for 4 h. The solution was placed on a steam still and made basic by introduction of an excess of 17 N NaOH through a septum. The ammonia was trapped in 0.1 N sulfuric acid and quantified by Nessler's assay at 425 nm. The ammonia was oxidized to nitrogen gas in vacuo using sodium hypobromite. The nitrogen gas produced was distilled on high vacuum lines and analyzed as described above.

Data Analysis. The abundance of ^{15}N in unreacted starting material (R_o), in remaining substrate (R_s), and product (R_p) were determined as described, as was the fraction of reaction *f*. For comparisons between R_o and R_s, the isotope effects were calculated using eq 1.

$$\text{isotope effect} = \log(1 - f)/\log[(1 - f)(R_s/R_o)] \quad (1)$$

For comparisons between R_o and R_p, the isotope effects were calculated using eq 2.

$$\text{isotope effect} = \log(1 - f)/\log[1 - f(R_p/R_o)] \quad (2)$$

RESULTS

Picolinic Acid and N-Methyl Picolinic Acid. Determination of heavy-atom isotope effects for these compounds followed effectively the same procedure. Reactions were performed in ethylene glycol so that solutions could be heated to 190 °C without resorting to the use of sealed systems and so that picolinic acid would be present to some degree as the neutral species as opposed to the zwitterion (*13*). For reactions with *N*-methyl picolinic acid, quinoline was used as a base so that the carboxyl group could be ionized and the product carbanion neutralized. The amount of carbon dioxide released was determined by manometry and compared to the amount of remaining substrate as determined by UV−vis assay to calculate the fraction of reaction. For carbon isotope effects, the abundance of ^{13}C in the carboxyl group of the starting material was determined by total decarboxylation of the substrate. This number was compared to the abundance of ^{13}C in the carbon dioxide released by partial reaction of the substrate to determine the isotope effects. For nitrogen effects, the abundance of ^{15}N in the starting material was determined by combustion of the starting material. With picolinic acid, the pyridine product could not be isolated without fractionation due to volatilization. Therefore, only the abundance of ^{15}N in the remaining starting material could be used to determine the isotope effects. With *N*-methyl picolinic acid, the abundance of ^{15}N in both the remaining substrate and the product could be determined, and two independent measurements were available for the nitrogen effects. The isotope effects were corrected for the elevated temperatures using eq 3

$$298[\ln({}^{x}k_{298})] = T[\ln({}^{x}k_{T})] \quad (3)$$

where ${}^{x}k_T$ is the isotope effect at temperature *T* in degrees K (Table 1) (*14*).

Orotidine 5′-Monophosphate. ^{15}N isotope effects on the enzymatic decarboxylation of orotidylate were determined using a degradative procedure to isolate the nitrogens of the nucleotide base without fractionation (Figure 2). The deg-

Table 1: ^{13}C and ^{15}N Decarboxylation Isotope Effects

	picolinic acid[a]		*N*-methyl picolinate[b]		OMP[c]
	^{13}k	^{15}k	^{13}k	^{15}k	$^{15}(V/K)$
measured effect	1.0156 ± 0.0005	0.9955 ± 0.0004	1.0212 ± 0.0002	1.0053 ± 0.0002	1.0036 ± 0.0003
corrected to 25 °C	1.0243 ± 0.0008	0.9930 ± 0.0006	1.0281 ± 0.0003	1.0070 ± 0.0003	1.0036 ± 0.0003

[a] Ethylene glycol, 190 °C.[b] Ethylene glycol with quinoline, 120 °C.[c] ODCase, pH 6.5, 25 °C.

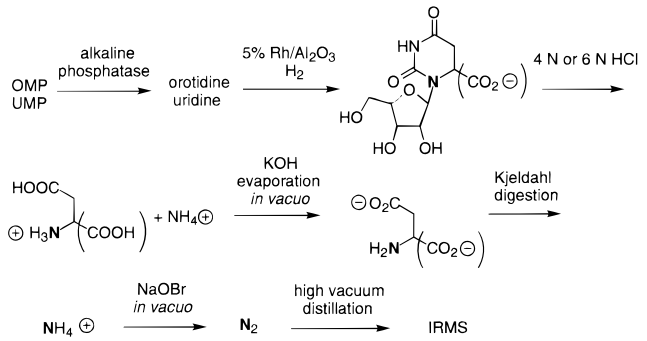

FIGURE 2: Degradation of OMP and UMP.

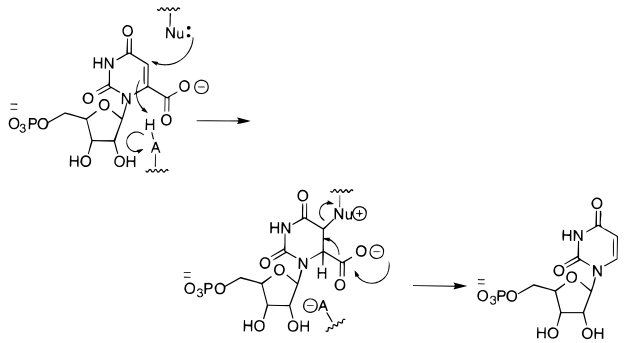

FIGURE 3: Covalent catalysis of decarboxylation of OMP.

radations of the substrate OMP and product UMP were essentially the same with one exception. The nucleotides were separated, and the phosphate was removed to allow complete desalting of the nucleosides by reversed-phase HPLC. This was essential to avoid poisoning the hydrogenation catalyst in the following step. The dihydronucleosides are susceptible to ring-opening of the reduced base in hot acid with specific hydrolysis of N-3 to give ammonia. However, dihydroorotidine is slightly more vulnerable to nonspecific digestion, resulting in some ammonia formation from N-1 as well, with concomitant fractionation. Thus, the acid hydrolysis conditions were slightly milder for dihydroorotidine. Proton NMR analysis of the product of the acid digestion showed complete hydrolysis of the reduced nucleosides. After titration of the sample to pH 11, the ammonia was volatilized, leaving N-1 in the form of an amino acid and allowing specific determination of the ^{15}N abundance at that position by Kjeldahl digestion. The amount of ammonia present after the HCl hydrolysis and after the Kjeldahl digestion was determined by Nessler's assay and found to be equal. Since NMR analysis showed complete reaction of the reduced nucleoside, the equivalence of the amount of ammonia after each acid digestion showed that the two nitrogens had been completely separated. The abundance of ^{15}N in the starting material was determined by degradation of samples of unreacted OMP, and this number was compared to the ^{15}N abundance of remaining starting material and product in partial enzymatic reactions, giving two independent determinations of the ^{15}N isotope effect per reaction (Table 1).

DISCUSSION

Decarboxylation reactions result in the formation of an anionic transition state or intermediate as a result of the neutralization of the negative charge on the carboxyl group. Catalysis results from the stabilization of this anionic species. In the case of ODCase, the substrate has no facile mechanism for delocalization of the negative charge. In addition, no cofactor or metal ions have been found to be necessary for catalysis by ODCase. The tremendous rate acceleration achieved by ODCase must therefore result only from interactions between enzymic functional groups and the substrate.

The formation of a covalent intermediate would provide a leaving group that could accept the negative charge that develops on decarboxylation (Figure 3). When substrate analogues with good leaving groups added to C-5 were prepared, nonenzymatic decarboxylation reactions resulted in the elimination of the leaving group and formation of a carbon−carbon double bond (*3*). However, when OMP was deuterated at C-5, no deuterium isotope effect on enzymatic decarboxylation was observed (*12*). Formation of a covalent intermediate at C-5 should result in a secondary deuterium effect of about 1.15 (*15*, *16*). In addition, when the barbituric acid 5′-monophosphate (BMP)−ODCase complex was studied by ^{13}C NMR, no change in the chemical shift of C-5 was observed from free BMP (*12*). If the incredibly tight binding of BMP [9×10^{-12} M (*17*)] were due to formation of a covalent adduct at C-5, then a change in the chemical shift at that position would be expected. If the extremely tight binding of BMP is due to resemblance to the transition state for decarboxylation, then this indicates that there is no covalent adduct in the transition state.

The carbanion formed on decarboxylation of OMP could be stabilized by an ylide intermediate with an adjacent positive charge on N-1. Several substrate analogues have been prepared that differed in their ability to form a positive charge at N-1. The analogues that were better able to form a positive charge adjacent to the site of decarboxylation showed a much greater rate of nonenzymatic decarboxylation (*4*, *18*). OMP is capable of forming a positive charge at N-1 by enolization. The enolized form could be stabilized by a low barrier hydrogen bond to a suitable side chain (Figure 4) (*5*). This preformed intermediate would stabilize a carbanion at C-6. A further possibility for stabilization of a carbanion at C-6 is the formation of a carbene at C-6 with transfer of a proton to O-4 in concert with decarboxylation (Figure 5) (*6*). The protonation of O-4 is, however, critical for this mechanism. Accordingly, several substrate analogues have been prepared with altered basicity of the oxygen contributing to the carbene structure. The rates of nonenzy-

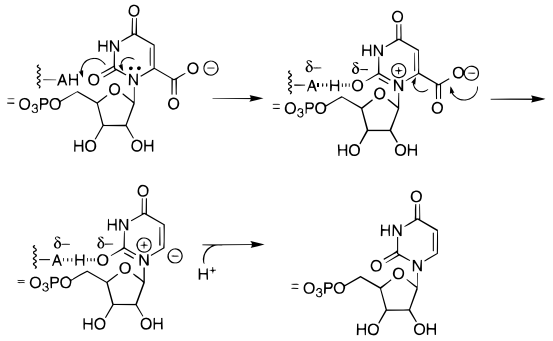

FIGURE 4: Catalysis of decarboxylation with an ylide intermediate.

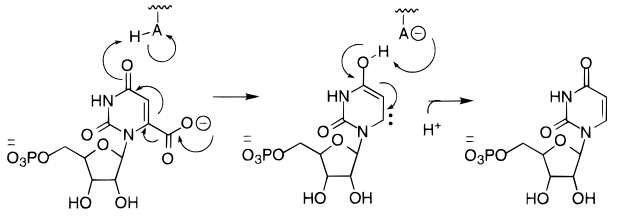

FIGURE 5: Carbene formation with protonation at O-4.

matic decarboxylation were found to depend on the basicity of the oxygen as well as the proximity of a ring nitrogen capable of forming an ylide (*7*). The reduction of the observed ^{13}C isotope effect with D_2O as the solvent led to the suggestion that protonation at O-4 occurred prior to decarboxylation in a mechanism involving a carbene (*19*). A reduction of the observed ^{13}C isotope effect with deuteration shows that a step other than decarboxylation must become more rate limiting with deuteration. This does not, however, prove that there is transfer of a proton to O-4 prior to decarboxylation (see below).

An important side chain in the catalytic mechanism is Lys-93. When this side chain was replaced by cysteine, no catalysis was observed, but rescue of the mutant with bromethylamine restored activity (*20*). A lysine side chain has a possible role in all of the proposed mechanisms. In the covalent mechanism, the lysine could add to C-5 after deprotonation. In the ylide mechanism, lysine could form a low barrier hydrogen bond to O-2. In the carbene mechanism, lysine could donate a proton to O-4. In each case, loss of the lysine would have a dramatic effect on catalysis.

Determination of ^{15}N isotope effects at N-1 of OMP can distinguish between mechanisms involving formation of a quaternary nitrogen and mechanisms where this nitrogen remains ternary. The ^{13}C isotope effects for decarboxylation are at least 1.0221 under any conditions (*11*), showing that the decarboxylation step is largely rate determining and any observed isotope effects will represent the kinetic effect for the decarboxylation multiplied by the equilibrium effect for the formation of any intermediate immediately prior to decarboxylation. If ODCase employs an ylide mechanism, then a large, inverse isotope effect for formation of a quaternary nitrogen intermediate will contribute to the observed ^{15}N effect. For comparison, protonation of pyridine shows an equilibrium ^{15}N isotope effect of 0.9793 (*21*).[2] Since any kinetic effect will be a secondary one, an equilibrium effect of this size should swamp the kinetic effect and result in an observed inverse ^{15}N isotope effect. If N-1 remains ternary prior to decarboxylation, then the equilibrium effect will be unity, and a normal isotope effect should be observed as a result of the loss of N−C−C bending and N−C−C−O torsional modes.

The ^{13}C and ^{15}N kinetic isotope effects for decarboxylation of picolinic acid and *N*-methyl picolinic acid in ethylene glycol were determined to provide a basis for comparison with a relatively simple system. The rate of decarboxylation of picolinic acid in ethylene glycol is much smaller than that of *N*-methyl picolinic acid, necessitating higher temperatures with the former. In both cases, the large ^{13}C isotope effects show that the decarboxylation step is highly rate determining, as expected. With picolinic acid, the inverse ^{15}N effect results from the formation of a zwitterion with transfer of the proton from the carboxyl group to the nitrogen. The inverse equilibrium isotope effect for this process is larger than any possible secondary kinetic isotope effect on decarboxylation. This result clearly indicates that decarboxylation takes place from a preformed zwitterion. Concerted proton transfer would result in a normal isotope effect from formation of a new bond to the nitrogen in the transition state as well as loss of the vibrational modes with the carboxyl group. Decarboxylation from the neutral species, with proton transfer to a second picolinic acid molecule, would also result in a normal isotope effect, as the nitrogen would remain ternary prior to decarboxylation. With *N*-methyl picolinic acid, the normal isotope effect observed results from the loss of the bending and torsional modes with the carboxyl group. In this case, there can be no equilibrium effect as the nitrogen is already quaternary in the ground state. Thus, the effects with *N*-methyl picolinic acid are very simplified and result only from the decarboxylation. The faster rate of decarboxylation with *N*-methyl picolinic acid is due to the positive charge that is present at N-1. With picolinic acid, the form that has a positive charge at N-1 is only a minor form at equilibrium.

The previously determined ^{13}C isotope effects for decarboxylation of OMP at pH 6.0 and 6.8 were 1.0272 and 1.0247, respectively (*11*). The ^{13}C effect at pH 6.5 should lie between these two values. The largest observed ^{13}C isotope effect was 1.049 at pH 4, where the decarboxylation reaction was most highly rate determining (*11*). Using 1.049 for the intrinsic isotope effect and 1.026 as the estimated ^{13}C effect at pH 6.5, the forward commitment factor c_f at pH 6.5 can be calculated from eq 4 as 0.885,

$$^{13}(V/K) = (^{13}k + c_f)/(1 + c_f) \qquad (4)$$

where $^{13}(V/K)$ is the observed isotope effect on V/K at pH 6.5 and ^{13}k is the intrinsic isotope effect. The intrinsic isotope effect represents the true effect of isotopic substitution on the rate of decarboxylation, while the forward commitment factor represents the reduction of the true isotope effect that takes place as a result of the tendency of the substrate to react forward once bound to the enzyme rather than dissociate. The calculated forward commitment can then be used with the observed ^{15}N effect in an equation similar to 4, but

[2] The protonation of pyridine is a better model for the increase in bond order at N-1 of OMP than the conversion of acetylpyridine-NADH to acetylpyridine-NAD ($^{15}K_{eq} = 0.9958$) (*22*), since there is considerable double bond character in the N1−C6 bond of acetylpyridine-NADH, as shown by the long wavelength band at 363 nm. In OMP, the keto form is thought to dominate and thus there is little positive charge on N-1.

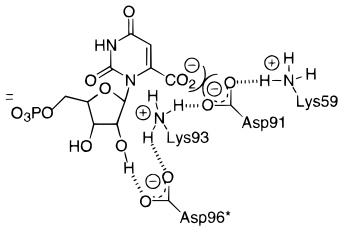

FIGURE 6: Binding of OMP to yeast ODCase based on structure with BMP. Electrostatic and steric interactions between Asp91 and substrate carboxyl destabilize ground state. Lys93 interacts with and protonates C-6 carbanion. The residue numbers are for the yeast enzyme (*8*).

with superscripts of 15, to calculate an intrinsic ^{15}N effect of 1.0068.[3] This value is nearly identical to that of *N*-methyl picolinic acid, clearly indicating that N-1 does not undergo any bond order changes prior to decarboxylation. This rules out the possibility of a preformed intermediate with a quaternary nitrogen.

Enzymatic catalysis of OMP decarboxylation appears to be accomplished by ground-state destabilization caused by electrostatic repulsion between the substrate carboxyl and a neighboring aspartate. Pai and co-workers determined the structure of OMP with 6-azaUMP bound in the active site and performed quantum mechanical calculations based on these data to address this question (*9*). The structure of 6-azaUMP bound in the active site revealed that the missing 6-carboxyl group would overlap or be in close contact with an aspartate side chain. The repulsive interaction between the carboxyl group and the aspartate side chain would result in strain, destabilizing the ground state of OMP. This aspartate was part of a Asp-Lys-Asp-Lys chain of interacting side chains containing the catalytic lysine. The catalytic lysine was correctly placed to interact with the carbanion formed at C-6 and protonate that carbon after cleavage of the carbon−carbon bond. Calculations showed that these interactions would provide the required energy to explain the tremendous rate acceleration achieved by ODCase (Figure 6) (*9*). These interactions do not involve formation of a quaternary nitrogen at N-1 and are therefore consistent with the observed ^{15}N isotope effects. Note that the catalytic lysine in the enzymatic reaction plays the role that a protonated N-1 plays in the nonenzymatic reactions and does so without the energetic cost of enolizing the substrate.

The remaining mechanistic question is the possible contribution of a carbene intermediate. Although consistent with a normal ^{15}N isotope effect at N-1, calculations showed that formation of a carbene requires transfer of a proton to O-4 (*6*). The recently published crystal structures show that there is no acidic side chain in the correct orientation for such a transfer, and that Lys-93 is in position to interact with C-6, not O-4 (*8*−*10*). Thus, at most, a carbene resonance form might make a minor contribution to the putative carbanion intermediate.

The decrease in the observed ^{13}C isotope effect with deuteration of the solvent (*19*) is presumably due to conformational changes prior to catalysis which become slower in both directions in D_2O, rather than to a transfer of a deuteron to O-4 prior to decarboxylation. This phenomenon has been observed with glucose-6-phosphate dehydrogenase where the forward commitment to hydride transfer was doubled in D_2O (*23*).

[3] A 20% error in estimating the forward commitment will cause the calculated intrinsic ^{15}N isotope effect to vary only between 1.0061 and 1.0074, which will not change the conclusions in this paper.

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