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Stabilities of Uracil and Pyridone-Based Carbanions: A Systematic Study in the Gas Phase and Solution and Implications for the Mechanism of Orotidine-5'-Monophosphate Decarboxylase

Nicholas A. Senger[†], Carly E. Bliss[†], James R. Keeffe[†], Scott Gronert^{‡,*}, and Weiming Wu^{†,*}

[†]Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, California 94132, USA

[‡]Department of Chemistry, Virginia Commonwealth University, 1001 W. Main Street, Richmond, Virginia 23284, USA

Abstract

The stabilities of the C6-centered carbanions derived from 1,3-dimethyluracil, N-methyl-2-pyridone, and N-methyl-4-pyridone were systematically investigated in the gas phase and in DMSO and water solutions. The stabilities of the carbanions in the gas phase and DMSO were directly measured through their reactions with carbon acids with known proton affinity or pK_a values. The stabilities of the carbanions in DMSO were also probed through their kinetic isotope effects of protonation over deuteration using acids with different acidity. The stabilities of the carbanions in water were determined through the rates of hydrogen-deuterium exchange reactions of the corresponding conjugate acids. The carbanions derived from the two pyridones were found to have the same stability, whereas the carbanion derived from 1,3-dimethyluracil was more stable. The order of the stability of the carbanions showed no correlation with the decarboxylation rates of their corresponding carboxylic acids. The implications of the results for the mechanism of orotidine-5'-monophosphate decarboxylase (ODCase) are discussed.

Keywords

pK_a ; carbanion stability; uracil; pyridone; isotope effect; ODCase

1. Introduction

The non-enzymatic decarboxylation of 1,3-dimethylorotic acid (**1**) and its structural analogues **2** and **3** to their respective uracil and pyridone products **4–6** as shown below has been studied as a model for the enzymatic decarboxylation catalyzed by orotidine-5 -

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*To whom correspondence should be addressed: Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132. Telephone: (415) 338-1436. Fax: (415) 338-2384. wuw@sfsu.edu Or at Department of Chemistry, Virginia Commonwealth University, 1001 West Main St, Richmond, VA 23284. Telephone: (804) 828-2753. Fax: (804) 828-8599. sgronert@vcu.edu.

Supplementary data Cartesian coordinates for computed structures in this study are available.

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monophosphate decarboxylase (ODCase).¹⁻¹⁵ Despite the structural similarity, acid **3** decarboxylates more readily than either acid **1** or **2** by a factor of approximately 1000.^{1,8} The stabilities of the presumed carbanion intermediates **7-9** have been investigated to understand the role they may have played in determining the rate of decarboxylation.

2. Results and Discussion

The stabilities of carbanions **7-9** have been studied in the gas phase, in water, and in dimethylsulfoxide (DMSO) in our laboratories.^{7,8,13,14} The gas phase study was carried out in a quadrupole ion trap mass spectrometer where the carbanions were generated via collision activated dissociation (CAD) of precursor carboxylate anions derived from acids **1-3** as the result of electrospray ionization.^{7,8} The carbanions were allowed to react with a series of neutral carbon acids with varying strength as seen in Table 1.¹⁶ The bracketing between the weakest acid that underwent a proton transfer and the strongest that did not undergo a proton transfer gave estimates of the strength of the carbanions as bases in terms of of PA values. Proton affinities of the carbanions were also calculated at the MP2/6-31+G(d,p)//HF/6-31+G(d) and B3LYP/6-31+G(d,p)//HF/6-31+G(d) levels to support the experimental work. The experimental and calculated values are in good agreement and the results are shown in Table 1.^{7,8}

The stabilities of carbanions **8** and **9** appear to be the same, whereas carbanion **7** is more stable by 7 kcal/mol. The identical stabilities of carbanion **9**, which is derived from the much more polar 4-pyridone, and carbanion **8** suggests that the contribution from the carbene resonance structure **9a** is quite limited.¹⁷ Overall, the carbanions are unusually stable for sp^2 hybridized carbanions. For comparison, the C^- -carbanion derived from pyridine has a proton affinity of 391 kcal/mol.¹⁶ Apparently the carbanions are stabilized by the carbonyl functional group. It has been suggested that uracil- or pyridone-based carbanions derive some stability from the adjacent N-C=O moiety.¹⁸ In the gas phase, carbanion **7** is as stable as an enolate, leading to the speculation that the presumed carbanion intermediate in the enzymatic decarboxylation may be quite stable if the active site provides a low-dielectric environment. In addition, the gas phase results makes it clear that the stabilities of the carbanions **7-9** are not correlated with the rates of decarboxylation of acids **1-3**. Thus, the relative stability of the carbanion intermediates does not play a major role in determining the rate of decarboxylation.

However, the results obtained in the gas phase may not be applicable in the condensed phase. Solvation usually plays a major role in the relative stability of molecules, especially ionic species. Solvation may alter the stability and the order of stability of the carbanions. The stabilities of carbanions **7–9** were therefore investigated in water and in dimethyl sulfoxide (DMSO).

In aqueous solution, the stabilities of carbanions **7–9** were compared by measuring the rate of the proton-deuterium exchange reaction as described by Equation 1.¹³ The rate of exchange is determined by the stability of the carbanion intermediate. The more stable the carbanion intermediate, the faster the exchange reaction.

To quantify the results, $\text{p}K_{\text{a}}$ values can be calculated from the rates of exchange using Equation 2.^{18,19} Richard and coworkers have utilized this method to determine the $\text{p}K_{\text{a}}$ values of the α -position of amino acids.^{18,19} The rate of exchange, k_{DO} , is converted to k_{HO} using the secondary solvent deuterium isotope effect of $k_{\text{DO}}/k_{\text{HO}} = 1.46$. In Equation 2, K_{w} is the water dissociation constant. k_{HOH} is the rate constant for the reaction between the carbanion and water and is assumed to be diffusion-controlled ($\sim 10^{11} \text{ s}^{-1}$) when the carbanion intermediate is very unstable as in the reaction of very weak carbon acids.

$$\text{p}K_{\text{a}} = \text{p}K_{\text{w}} + \log(k_{\text{HOH}}/k_{\text{HO}}) \quad (2)$$

The same method was used to determine the $\text{p}K_{\text{a}}$ of uracil **4** where the rate of exchange was determined at high temperatures (175–217 °C) in an acetate buffer in D_2O .⁹ However, when pyridones **5** and **6** were treated under the same conditions, no proton-deuterium exchange was observed, indicating that carbanions **8** and **9** are less stable than carbanion **7**. The rates of proton-deuterium exchange were eventually determined over the temperature range of 80–110 °C in 1 N NaOD/ D_2O and the results are summarized in Table 2. The use of NaOD as a base provides the advantage that correction of the ionization constants of the buffer over the temperature range is no longer required, eliminating a source of possible uncertainty. The results show the same trend observed in the gas phase and again indicate that the stability of carbanion intermediates does not play a major role in the decarboxylation of acids **1–3**.

The activation parameters for the proton-deuterium exchange reactions of pyridones **5** and **6** were also measured (the same study was not carried out with uracil **4** due to the presence of the hydrolysis of the uracil moiety as side reaction).^{13,20} The parameters were calculated at 298 K from the Eyring equation using data from four temperatures (80, 90, 100, and 110 °C, $r^2 = 0.99$).

The relative stabilities of carbanions **7–9** were also studied in DMSO, which has properties similar to the solvent used for the kinetic study of the decarboxylation reactions, sulfolane. In DMSO, both the thermodynamic and the kinetic stability of these carbanions were probed. In one set of experiments, carbanions **7–9** were generated through the decarboxylation of their corresponding carboxylates at 230 °C in DMSO and were allowed to react with weak carbon acids of varying strength as shown in Table 3.¹⁴ The weak acids in Table 3 were chosen for the range of $\text{p}K_{\text{a}}$ values, and most importantly, for the fact that their conjugate bases (carbanions) are colored.²¹ These weak acid thus act as indicators in this experiment.

The bracketing method is essentially the same as the one utilized in the gas phase study. Theoretically, carbanions **7–9** can also be generated through the deprotonation of the corresponding neutral species **4–6** by a strong base. Unfortunately this approach is found to be impractical due to the preferential deprotonation of the methyl group in pyridone **5**.²² If carbanion **7** or **8** or **9** is a strong enough base, it will deprotonate the indicator weak acid (In–H) and produce a colored carbanion (In[–]) as a conjugate base as shown in Equation 3. The bracketing between the strongest acid that yielded a color change and the weakest acid that did not yield a color change gives estimates of the p*K*_a values. DMSO has a reported p*K*_a of 35 so it is not a strong enough acid to protonate carbanions **7–9**.²¹

The acid-base equilibria were also reached from a different direction, in which conjugate bases of these indicators were allowed to react with uracil **4** and pyridones **5** and **6**. The indicators were treated with butyllithium to generate the conjugate bases as colored anions. The ability of molecules **4–6** to quench the color (as shown in Table 4) allows the bracketing of their acidity in the form of p*K* values.¹⁴ The deprotonation of the methyl group in pyridone **5** may also be an issue here, but this has no effect on the observation that uracil **4** is more acidic than pyridones **5** and **6**.

The results from the equilibrium between carbanions **7–9** and indicators demonstrate the same trend of stability in DMSO as found in the gas phase and water. To further investigate the relative stability of the carbanions, the stabilities of carbanions **7** and **8** were also compared by a kinetic method. In this experiment, decarboxylations of acids **1** and **2** were carried out in DMSO in the presence of a weak acid as the proton or deuterium donor. In the experiment, three acids with varying acid strength (as judged by their p*K*_a values in DMSO) were employed.²¹ Trifluoroethanol is much more acidic than uracil **4** or pyridones **5** and **6**; while the acidity of water and acetonitrile should be similar to **4–6** in DMSO. The results are summarized in Table 6. No isotope effect was observed when trifluoroethanol and trifluoroethanol-*d* were used as proton donors. The results obtained with H₂O/ D₂O and CH₃CN/CD₃CN as proton or deuterium donors show a large isotope effect for the protonation of carbanion **7**. For example, the isotope effect for the protonation over deuteration of carbanion **7** by CH₃CN/CD₃CN is 6.7, a clear primary isotope effect. On the other hand, the isotope effects for the protonation of carbanion **8** are much smaller.

If the carbanion produced from the decarboxylation is much more basic than the conjugate base of the weak acid, the protonation step will be diffusion-controlled and thus will not discriminate between the proton or deuterium ions. Therefore, no isotope effect will be observed under such conditions, just as seen with trifluoroethanol as the proton donor. However, when the basicity of the carbanion produced is similar to the conjugate base of the acid, the protonation of the carbanion will be more favorable than deuteration; an isotope effect will thus be observed.

To examine the interactions of the carbanions with acetonitrile, the proton affinities for carbanions **7** and **8**, as well as for the conjugate base of acetonitrile, were also calculated at the G3MP2 level to be 366.9, 374.4, and 373.2 kcal/mol at 298 K, respectively. The calculated values for carbanions **7** and **8** are very similar to the values reported in Table 1. The calculated value for the conjugate anion of acetonitrile is in good agreement with the experimental value of 372.9 kcal/mol.¹⁶ Therefore, the proton transfer reaction from acetonitrile to carbanion **7** is slightly exothermic; whereas that from acetonitrile to carbanion **8** is endothermic as shown in Table 6.

The kinetic isotope results obtained with **7** and **8** are consistent with two interpretations. One simple explanation is that the protonation of carbanion **8** is close to the diffusion-controlled limit due to its stronger basicity and thus the rates for hydrogen and deuterium abstraction converge to this limit. The other explanation is that transition state for the proton transfer reaction for the stronger base is less symmetric and for that reason produces a smaller isotope effect. Either way, the results suggest that carbanion **7** is less basic than carbanion **8**, in agreement with the results from the equilibrium studies discussed above.

The stabilities of carbanions that are possible intermediates in the decarboxylation of orotic acid and its analogues have been determined in different media (gas phase, water, and DMSO) and with different methods (computational, kinetic and thermodynamic) as described above and the results are summarized in Table 7. All the results suggest that the uracil-based carbanion **7** is more stable than carbanions **8** and **9**, which are derived from its 2-pyridone and 4-pyridone analogues. Carbanions **8** and **9** have almost identical stability in all media. However, the rate constant for the decarboxylation of acid **3** is approximately 1000 times faster than that of either acid **1** or **2** (Table 7).^{1,8} Therefore, there is a lack of correlation between the rate of decarboxylation and the stabilities of the carbanion intermediates. This lack of a correlation indicates that the non-enzymatic decarboxylation of orotic acid analogues is unlikely by a mechanism in which the loss of CO₂ results in the direct formation of carbanion intermediates.

We have proposed a step-wise mechanism for the model decarboxylation of orotic acid analogues as illustrated in Scheme 1 using 1,3-dimethylorotic acid as an example.^{1,8} This mechanism envisions a rapid equilibrium between the acid (e.g. **1**) and a zwitterionic structure (e.g. **10**) followed by the loss of CO₂. In the rate equation derived for this mechanism, the observed rate constant k_{ob} is the product of the equilibrium constant for the first step, K , and the rate constant for the second step, k . Therefore, k_{ob} is proportional to the equilibrium constant K .^{1,8} The population of the zwitterionic structures such as **10** determines the rate of the reaction.

To test the assumptions that underlie the proposed mechanism, we have investigated the energetics of both reaction steps. We have previously reported that the oxygen atom of the ring carbonyl group in acid **3** is much more prone to protonation than those in acid **1** and **2**.^{7,8} We have also calculated the energy difference between structures **1** and **10** in Scheme 1 and among corresponding structures for acids **2** and **3** at the G3MP2 level as shown in Scheme 2. Comparison of the energy differences between acids **1**, **2**, and **3** and their zwitterions shows that zwitterion **13** from acid **3** is much more favored than its counterparts **10** and **12**. Interestingly the equilibria between **1** and **10** as well as **2** and **12**, respectively, are about equally positioned. These results correlate well with the kinetic results in Table 7 showing that acid **3** decarboxylates much faster than either acid **1** or acid **2**, which have nearly the same rate constants.

We have further calculated the energy barriers at the G3MP2 level for the second step of the proposed reaction mechanism, the loss of CO₂, and the results are shown in Scheme 3. The calculated energy barriers are very similar, providing support for our assumption that the loss of CO₂ after the formation of the zwitterionic carboxylates has similar rates in the overall decarboxylation reactions of acid **1**–**3**. We have also calculated the overall energies of the two-step transformations from **1** to **11**, **2** to **14**, and **3** to **15** as envisioned by the proposed mechanism. The energies for the three transformations are 39.3, 37.5, and 24.6 kcal/mol, respectively. The transformation from **3** to **14** is indeed much more favorable by more than 10 kcal/mol. Our calculations also indicated that O-4 is the preferred site of protonation, the energy barrier for the decarboxylation of acid **1** with O-2 protonation is a much less favorable 54.8 kcal/mol, in good agreement with reported results.⁴

3. Conclusion

Our experimental and theoretical studies on the model decarboxylation of orotic acid derivative **1** and pyridone acids **2** and **3** and the reactive intermediates of these reactions have demonstrated a lack of correlation between the stability of the carbanion intermediate and the rate of decarboxylation of corresponding acid. On the other hand, the results have provided strong support for the zwitterionic mechanism outlined in Scheme 1. However, it should be emphasized that the proposed mechanism is for the model and non-enzymatic decarboxylations. Whether the enzymatic reaction follows the same mechanism remains to be seen. Studies by Richard and coworkers have indicated the existence of a discrete carbanion-like intermediate in the enzymatic reaction.²³⁻²⁵ ODCase does not seem to contain protonating residues to allow the protonation of the carbonyl groups of the substrate.²⁶⁻³⁰ However, it has been hypothesized that a large dipole moment in the active site can accomplish the same effect (as schematically shown below).³¹⁻³⁴ We have further suggested that substrate analogues that favor the formation of the zwitterionic structures are bound more tightly by ODCase.³¹⁻³³ We are currently probing the structures of the substrate and its analogues to determine whether there is a correlation between their zwitterionic content and their binding by ODCase.

4. Experimental Methods

The experimental methods of some of the studies have been reported earlier.^{7,8,13,14}

Chemicals

Deuterium oxide, 2,2,2-trifluoroethanol-d₃, and acetonitrile-d₃ are commercially available from Cambridge Isotope Laboratory. Acid **1** was prepared through the methylation of orotic acid (from Sigma) as reported by Curran and Angier.³⁵ The same method was followed to prepare **2** from 2-pyridone-6-carboxylic acid (from Aldrich). Acid **3** was prepared from 4-pyridone-2-carboxylic acid (from TCI America) as reported by Beak and Siegel.¹ The identity and purity of the synthesized compounds was verified by their ¹H NMR spectra and melting points.

Calculations

All calculations were completed at the G3MP2 level using the GAUSSIAN 03 program.³⁶ When appropriate, multiple rotamers were probed at lower levels of theory and the most

stable was submitted to the G3MP2 calculations. Values were reported as enthalpies at 298 K unless indicated otherwise.

Kinetic Isotope Studies in DMSO

Typical procedure using CH₃CN/CD₃CN as donors. Acid **1** or **2** (about 10 mg), CH₃CN (about 25 mg), and CD₃CN (about 90 mg) were weighed to 0.1 mg and mixed with DMSO-d₆ (0.75 mL) in a NMR tube. This can be more conveniently done by preparing a stock solution of proportional amount of CH₃CN and CD₃CN in DMSO-d₆. The tube was heated at 190 °C for 3–4 hrs. In the reaction with acid **2**, the NMR spectrum of this reaction mixture was recorded upon cooling. The integration values of H-6 and H-4 of the product were employed to calculate the ratio of hydrogen and deuterium incorporation using the formula $H/D = [\text{integration of H-6}]/[(\text{integration of H-4}) - (\text{integration of H-6})]$.

In the reaction with acid **1**, the NMR spectrum of this reaction mixture was recorded after the addition of *p*-xylene (about 20 mg) measured to 0.1 mg as an external standard. The integration values of the NMR absorptions of H-6 and *p*-xylene were used to calculate the amount of unreacted acid and the amount of hydrogen-incorporated product. The amount of deuterium-incorporated product was determined by subtract the moles of hydrogen-incorporated product from the moles of acid consumed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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$$dP/dt = Kk[1]/(K+1) \quad \text{When } K \ll 1, k_{ob} = Kk$$

SCHEME 1.

SCHEME 2.

SCHEME 3.

Table 1Proton Affinities^a of Carbanions **7–9**

Reference acids	H _{acid}	Carbanion 7	Carbanion 8	Carbanion 9
Butanone	367.2 ± 2.8	yes	—	—
Acetone	369.1 ± 2.1	yes	—	—
Methacrylonitrile	370.7 ± 2.1	no	yes	yes
Propionitrile	375.0 ± 2.1	no	yes	yes
Butanol	375.4 ± 2.1	—	yes	yes
Propanol	375.7 ± 1.3	—	yes	yes
Ethanol	378.7 ± 1.0	—	yes but slow	yes but slow
Toluene	380.6 ± 1.0	—	no	no
Bracketed PA (300 K) Calculated PA (0 K) ^b		369.9 ± 3.1 367.6 (366.0)	377.0 ± 2.9 375.5 (374.0)	377.0 ± 2.9 375.8 (375.0)
Calculated PA (0 K) ^b		367.6 (366.0)	375.5 (374.0)	375.8 (375.0)

^a All values are in kcal/mol.^b Calculated at the MP2/6-31+G(d,p)//HF/6-31+G(d) level. Calculated values at the B3LYP/6-31+G(d,p)//HF/6-31+G(d) level are given in parentheses.

Table 2

H-D Exchange Reaction at Carbon-6 of Uracil and Pyridones

Substrates	Uracil 4	Pyridone 5	Pyridone 6
k_{OH} ($\text{M}^{-1} \text{s}^{-1}$)	$\sim 3 \times 10^{-6}$	3.31×10^{-8}	8.35×10^{-8}
$\text{p}K_{\text{a}}$	~ 30	32 ± 1	32 ± 1
$G^{\ddagger}$ (kcal/mol)	—	27.4	26.9
$H^{\ddagger}$ (kcal/mol)	—	21.2	20.2
$A^{\ddagger}$ (cal/mol/K)	—	-21.1	-22.8

Table 3Reactions between carbanions **7-9** and indicators in DMSO^a

Indicators	pK _a ^b	Carbanion 7	Carbanion 8	Carbanion 9
pentamethylcyclopentadiene	26.1	yellow	—	—
9-phenylxanthene	27.9	red	—	—
4-benzylpyridine	26.7	red	—	—
2-benzylpyridine	28.2	red	—	—
triphenylmethane	30.6	no change	red	red
diphenylmethane	32.2	no change	no change	red (slow)
Estimated pK _a values ^c		29 ± 1	31 ± 1	~32

^aReactions carried out at 230 °C.^bTaken from ref 21.^cThe pK_a values are those of corresponding conjugate acids (uracil 4 and pyridones 5 and 6).

Table 4Reactions of indicator carbanions with uracil **4** and pyridones **5-6** in DMSO^a

Indicators	Color of Anions	pK _a ^b	Uracil 4	Pyridone 5	Pyridone 6
4-benzylpyridine	red	26.7	red	—	—
2-benzylpyridine	red	28.2	red	—	—
triphenylmethane	red	30.6	quenched	red	red
diphenylmethane	orange	32.2	—	quenched	quenched
Estimated pK _a values			29 ± 1	31 ± 1	31 ± 1

^aReactions carried out at room temperature.^bTaken from ref 21.

Table 5Kinetic isotope effect in the protonation of carbanions **7** and **8** by various donors

Presumed carbanion intermediates	Ratio of H/D Incorporation in Various H/D Donors		
	CF ₃ CH ₂ OH/CF ₃ CH ₂ OD p <i>K</i> _a =23.45 ^a	H ₂ O/ D ₂ O p <i>K</i> _a =31.2 ^a	CH ₃ CN/CD ₃ CN p <i>K</i> _a =31.3 ^a
7 (experimental)	1.00±0.01	1.39±0.06	6.71±0.28
8 (experimental)	1.00±0.01	1.11±0.09	2.80±0.06

^a p*K*_a values take from Ref. 21.

Table 6Gas phase enthalpies and free energies for deprotonation of acetonitrile by carbanions **7** and **8**, kcal/mol.^a

Carbanions	H	G
7	6.4	5.6
8	-1.1	-2.0

^aEnthalpies and free energies were calculated at the G3MP2 level at 298 K.

Table 7Rate constants, proton affinities, and pK_a values

	uracil derived species	2-pyridone derived species	4-pyridone derived species
Rate constants for the decarboxylation of acids 1–3 at 206 °C (s^{-1})	7.5×10^{-4} ^a 1.6×10^{-3} ^b	1.2×10^{-3} ^a 1.3×10^{-3} ^b	0.32^a , 1.6^b
Gas-phase Stability of carbanions 7–9 (kcal/mol)	370	377	377
pK_a of 6-CH (4–6) in water	30	32	32
pK_a of 6-CH (4–6) in DMSO	29 ± 1	31 ± 1	31 ± 1

^aRate constants in sulfolane reported in ref 8.^bRate constants in isoquinoline reported in ref 8.