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The gas phase proton affinity of uracil: measuring multiple basic sites and implications for the enzyme mechanism of orotidine 5′-monophosphate decarboxylase

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We have shown for the first time experimentally that the O2 and O4 sites of uracil have different proton affinities, and as implied in previous computational studies, the O4 is more basic and would be energetically preferred in an orotate ribose 5′-monophosphate decarboxylase catalysis mechanism involving proton transfer to oxygen.

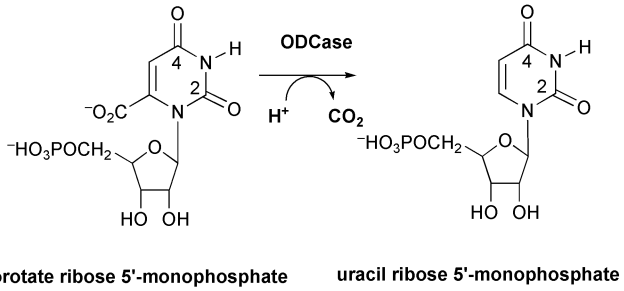
Orotidine 5′-monophosphate decarboxylase (ODCase) catalyzes the decarboxylation of orotate ribose 5′-monophosphate to form uracil ribose 5′-monophosphate (Scheme 1) and is the last step in the *de novo* synthesis of pyrimidine nucleotides. The catalytic mechanism does not utilize cofactor or metal ions and remains elusive. ODCase is an unusually proficient enzyme, with a $k_{cat}/k_{uncat}/K_m$ of 10^{23}, which indicates that the enzyme should be particularly susceptible to transition state analogs as inhibitors.[1–4]

One primary mechanistic proposal for ODCase catalysis involves proton transfer to either the 2 or 4 oxygen to promote decarboxylation. Although recently published crystal structures have raised concerns regarding protonation as a catalytic mechanism, no current hypothesis is wholly consistent with all existing data and proton transfer remains of interest. Quantum mechanical calculations suggest that O4-protonation followed by decarboxylation is preferred energetically over 2-protonation.[5–7] The calculations indicate that the energetic favorability for the O4-protonation mechanism lies in part in the greater basicity of the 4-oxygen over the 2-oxygen in orotate, in the transition state, and in uracil. Gas-phase calculations predict a 9 kcal mol^{-1} gap between the proton affinity (PA) of the more basic O4 and the less basic O2 site of uracil.[8] The experimental gas phase PA of the two oxygen sites of uracil has not heretofore been experimentally determined; we sought to measure the PAs to establish the intrinsic favorability of 4-oxygen protonation over 2-oxygen protonation.

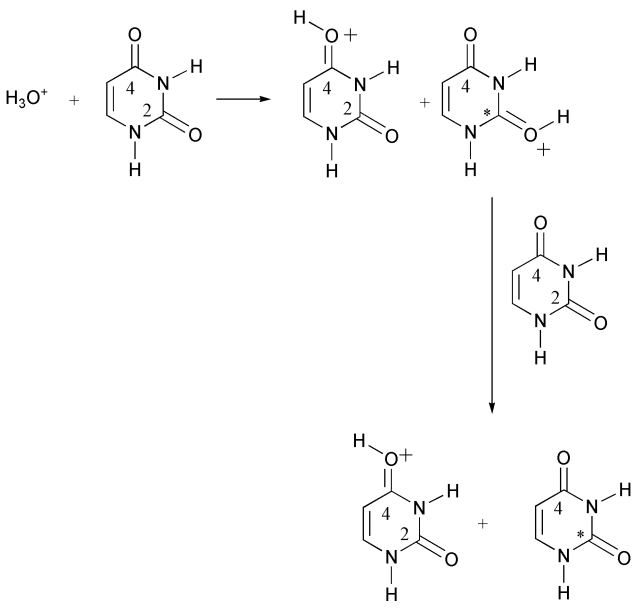
First, the PA of the more basic site of uracil was bracketed in the gas phase. The reaction of uracil with 2,4-pentadione (PA (2,4-pentadione) = 208.8 kcal mol^{-1}) proceeds in both directions: protonated uracil protonates 2,4-pentadione, and *vice versa*.[9,10] Protonated uracil does not protonate 2-methylfuran (PA (2-methylfuran) = 207.0 kcal mol^{-1}), but protonated 2-methylfuran does protonate uracil. Protonated pyrrole (PA (pyrrole) = 209.2 kcal mol^{-1}) does not protonate uracil, but the reverse reaction occurs. We therefore bracket the most basic site of uracil to be 209 ± 3 kcal mol^{-1}. Our result is in agreement with a previous experimental estimate of 204–215 kcal mol^{-1}.[11] Furthermore, Sindona *et al.* and Meot-Ner have measured the PA of thymine, which is the 5-methyl derivative of uracil, to be 210.9 and 209.0 kcal mol^{-1}, respectively.[12,13] Given that calculations predict that the O4 is the more basic site, we believe that it is the O4 that we have bracketed.[8]

Measuring the less basic site of a molecule is an experimental challenge. We have recently developed a dual cell Fourier transform mass spectrometric (FTMS) method for the bracketing of the less acidic sites in molecules that have multiple acidic sites.[14] We should in principle be able to apply this method to PA bracketing of less basic sites. In brief, when H_3O^+ (PA (water) = 165.2 kcal mol^{-1}) is used to protonate uracil, two ions should be formed, the O2-protonated ($O2H^+$) and the O4-protonated ($O4H^+$) uracil. When the ions are allowed to stay in an environment where there is a constant pressure of neutral uracil, the $O2H^+$ ion reacts with another molecule of uracil and isomerizes to $O4H^+$ (Scheme 2). In order to bracket the less basic site, we must transfer the $O2H^+/O4H^+$ mixture out of the neutral environment, into a second reaction cell, where the reference base is added at a constant pressure. Under these 'less basic' conditions, the $O2H^+$ will not completely isomerize to $O4H^+$, and the $O2H^+$ can be bracketed.

We find, however, that using this method, we cannot accurately measure the PA of the less basic O2 site of uracil. We are unable to pinpoint the lower limit of the proton affinity, because essentially, proton transfer is *always* detected. For example, we see proton transfer when the protonated uracil ions are allowed to react with acetic acid (PA (acetic acid) = 187.3 kcal mol^{-1}). Given that the proton affinity of the O4 site of uracil is 209 kcal mol^{-1}, and that calculations predict that the O2 site will be just 9 kcal mol^{-1} less basic, it would be unlikely



Scheme 1



Scheme 2

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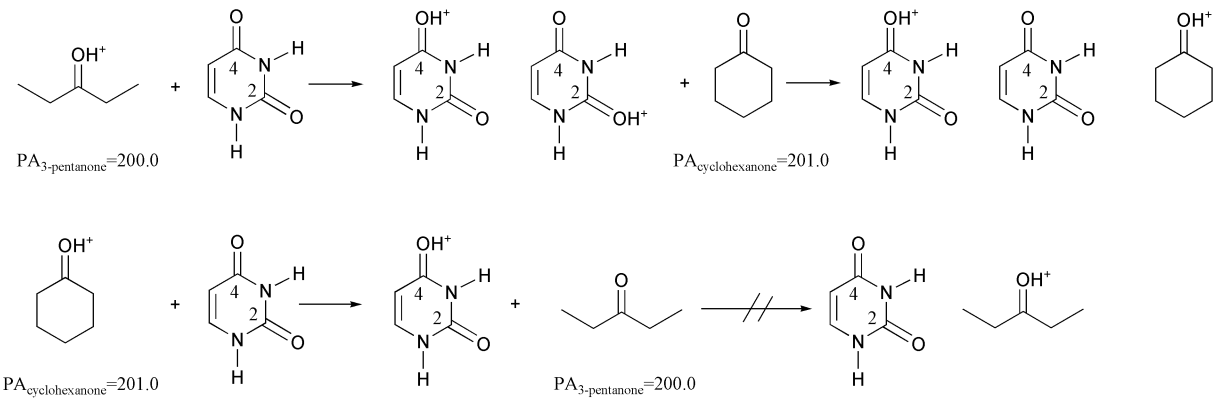

Scheme 3

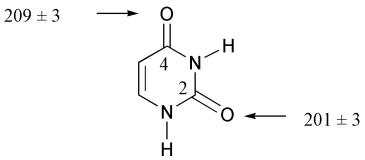

Fig. 1 The gas phase proton affinities of the O2 and O4 positions of uracil in kcal mol^{-1}.

that acetic acid could deprotonate O2H$^+$-uracil. We suspect that the constant proton transfer is either the result of an inability to completely eject the highly acidic H_3O^+, which when present will proton transfer to any of our reference bases, and/or insufficient cooling of the generated ions. To overcome these problems, we modified our experimental design to use a less acidic precursor ion to generate protonated uracil.

In Scheme 3 we show the modified experiment, where H_3O^+ (PA (water) = 165.2 kcal mol^{-1}) is used to protonate a known base, 3-pentanone. We isolate the 3-pentanoneH$^+$ ion (PA (3-pentanone) = 200.0 kcal mol^{-1}) and use it as our precursor ion to protonate uracil. The O2H$^+$/O4H$^+$ mixture is then transferred to the second cell, out of the neutral uracil environment, where the reference base is cyclohexanone. The PA of cyclohexanone is 201.0 kcal mol^{-1}. If there is any O4H$^+$ present, cyclohexanone will be unable to deprotonate it, because the PA of the O4 of uracil is 209 kcal mol^{-1}. Therefore, if proton transfer occurs, it must be the reaction between cyclohexanone and the O2H$^+$ ion. We do detect proton transfer, indicating that the uracil O2 site has a basicity *less than* 201.0 kcal mol^{-1}. Also, since the O2 site is protonated by 3-pentanoneH$^+$, it must be more basic than 200.0 kcal mol^{-1}. We also performed the experiment in the opposite direction, where we used protonated cyclohexanone as the precursor ion (PA (cyclohexanone) = 201.0 kcal mol^{-1}). Protonated cyclohexanone does react with uracil to produce the [M + H]$^+$ ion. However, when the protonated uracil is allowed to react with 3-pentanone (PA (3-pentanone) = 200.0 kcal mol^{-1}) proton transfer does not occur. As a 'back-up' bracketing experiment, we also utilized protonated crotonaldehyde (PA (crotonaldehyde) = 198.6 kcal mol^{-1}), which is slightly more acidic than protonated 3-pentanone, as the acid, and 4-heptanone (PA(4-heptanone) = 202.0 kcal mol^{-1}), which is slightly more basic than cyclohexanone, as the base. Under these conditions, we also see proton transfer, which confirms our initial bracketing result. We therefore bracket the less basic site of uracil to be between 3-pentanone and cyclohexanone, at 201 ± 3 kcal mol^{-1}.

A summary of the gas-phase experimental proton affinity results for the O2 and O4 sites of uracil are shown in Fig. 1. We have found that the two oxygen sites of uracil are differentiable, with the O4 being more basic than the O2, by 8 kcal mol^{-1}. These experimental results provide evidence for the intrinsically higher basicity of the O4 predicted by computations. This result has implications for the ODCase catalytic mechanism; since intrinsically the O4 is more basic, protonation at that site might be favored in the nonpolar active site of the enzyme. We have also developed a method by which multiple basicities can be measured using FTMS. This method allows the bracketing of the less basic O2 site in one experiment, using two different reference bases. Future studies will focus on generalizing the multiple-basicity bracketing experiment through examination of other DNA bases.

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