^{18}O Isotope Effects Support a Concerted Mechanism for Ribonuclease A

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Ribonuclease A catalyzes the cleavage of RNA in a two-step process. The phosphodiester bond is cleaved to yield a RNA strand with a free 5′ OH and another RNA strand with a 2′, 3′ cyclic monophosphate at the 3′ end. This cyclic intermediate is then hydrolyzed with enzymatic catalysis.[1] Classically, the first step of cleavage of RNA by ribonuclease A has been reported to proceed via a concerted mechanism in which His-12 acts to deprotonate the nucleophilic 2′ OH and His-119 acts to protonate the leaving group.[2] Chemical modification studies,[3] pH-rate studies,[4] and site directed mutagenesis studies of His-12 and His-119[5] are consistent with general acid−base catalysis. However, the mechanism has become the subject of much debate as a result of the proposal of a phosphorane intermediate in the catalytic mechanism. This concept was based on data obtained in model studies utilizing cyclodextrin−bis(imidazole) compounds[6] or imidazole or morpholine buffers as catalysts.[7] We report here a direct study of the enzymatic reaction catalyzed by ribonuclease A to test for the presence of a phosphorane intermediate.

We have measured heavy atom isotope effects on the ribonuclease A catalyzed cleavage of the alternate substrate uridine 3′-*m*-nitrobenzyl phosphate. This compound was chosen because (1) the pK_a of the *m*-nitrobenzyl alcohol leaving group (estimated pK_a = 14.9)[8] is close to that of a natural substrate nucleotide leaving group (estimated pK_a = 14.8),[5] suggesting that the chemistry involved in the cleavage events will be similar; (2) the V_m/K_m of substrate cleavage to cyclic UMP and *m*-nitrobenzyl alcohol is 10^4 less than for cleavage of UpA and 100-fold less than for cleavage of uridine 3′-*p*-nitrophenyl phosphate[5] (although the pH profile has the same bell shape as that for UpA) facilitating observation of the chemical rate limiting step; and (3) the leaving group contains a single nitrogen atom which can be used as a remote label. The decrease in the measured rate of cleavage of this substrate may be largely due to unproductive binding since the nucleotide binding specificity is lost at the leaving group with this substrate. Unproductive binding would have no effect on the reported V_m/K_m isotope effects.

Substrate was synthesized with ^{18}O at the bridge position or nonbridge positions of the phosphodiester bond with ^{15}N at the nitro group of the benzyl ring.[9] This was mixed with the corresponding compound with ^{16}O at the bridge position or nonbridge positions and ^{14}N in the nitro group to produce a mixture having the natural abundance of ^{15}N in the nitro group. The mixture was used for heavy atom isotope effect measurements using the internal competition method.[10,11] The results are shown in Figure 1.[12]

The internal competition method used in these experiments measures the isotope effect on V_m/K_m for the reaction. If a chemical step prior to the first irreversible step is rate limiting, the full intrinsic isotope effect will be observed on that chemical step. If a nonchemical step is entirely or partially rate limiting, the intrinsic isotope effect due to the chemical step will be masked by a commitment factor. A change in the pH of a reaction away from the optimum can serve to reduce such a commitment and increase the magnitude of the observed isotope effect by slowing down the chemical step and making it more rate-limiting. In the absence of a commitment, the intrinsic isotope effect on the chemical step will be observed at the optimum pH, and the magnitude of this effect will not change with the pH of the reaction. The constant large primary isotope effect (Figure 1) observed at the pH optimum (pH 5.0) and well away

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(9) ^{14}N and ^{15}N-*m*-nitrobenzyl alcohols were prepared by nitration of benzaldehyde with ^{14}N or ^{15}N -labeled ammonium nitrate in sulfuric acid, followed by reduction of the nitrobenzaldehyde with sodium borohydride in methanol. For preparation of the [bridge-^{18}O, ^{15}N]-labeled substrate, [^{18}O, ^{15}N]- benzyl alcohol was prepared by esterification of ^{15}N- *m*-nitrobenzyl alcohol with [$^{18}O_2$]-labeled benzoic acid (prepared from benzoyl chloride and $H_2{}^{18}$O) under Mitsunobu conditions (Mitsunobu, O. *Synthesis* **1981**, 1) followed by hydrolysis. The double labeled substrate was then prepared by reaction of 2′,5′-di-*tert*-butyldimethylsilyl uridine (Hakimelahi, G. H.; Proba, Z. A.; Ogilvie, K. K. *Can. J. Chem.* **1982,** *60*, 1106) with 2-cyanoethyl diisopropylchlorophosphoramidite with triethylamine and dimethylamino pyridine (DMAP) in THF, followed by reaction with [^{18}O,^{15}N] benzyl alcohol and tetrazole in acetonitrile, and then oxidation with *tert*-butylhydroperoxide. Deblocking was achieved by treatment with 1,8-diazabicyclo-[5.4.0]undec-7-ene followed by tetrabutylammonium fluoride. The nonbridge labeled substrate was prepared by the sequence (a) reaction of 2′,5′-di-*tert*-butyldimethylsilyl uridine with bis(diisopropylamino)chlorophosphine with triethylamine and DMAP in THF, (b) reaction of this intermediate with ^{15}N- *m*-nitrobenzyl alcohol and tetrazole in acetonitrile, and (c) reaction with methanol-^{18}O and tetrazole in acetonitrile, followed by oxidation with iodine/$H_2{}^{18}$O/collidine. Deblocking was achieved by reaction with *tert*-butylamine followed by tetrabutylammonium fluoride.

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(11) Reactions were performed at room temperature in succinate (pH 5.0) or Tris (pH 8.0) buffer. Reaction progress was monitored via calibrated HPLC analysis of a quenched reaction aliquot. Aliquots were quenched by addition to 45 mM benzyl alcohol (internal standard) in 0.1 M citrate, pH 2.5, in 10.8% methanol and separated on a Microsorb-MV C18 HPLC column (5 μm, 4.6 mm ID × 25 cm) equilibrated with 6 mM sodium phosphate, pH 7.0, in 27% methanol, followed by detection at 254 nm and peak integration. Reactions were allowed to proceed to 25%−50% completion, titrated to pH 7 (for succinate reactions) or pH 12.5 (for Tris reactions), and immediately extracted with diethyl ether to separate *m*-nitrobenzyl alcohol from substrate. HPLC analysis of ether and aqueous layers confirmed complete separation under these conditions. The residual substrate present in the aqueous layer was then cleaved with excess ribonuclease A to yield *m*-nitrobenzyl alcohol. *m*-Nitrobenzyl alcohol isolated from the partial reaction or from residual substrate cleavage was purified by sublimation. Nitrogen gas was liberated from purified product by combustion over copper oxide in a sealed tube and analyzed by isotope ratio mass spectrometry as described by Hengge, A. C.; Edens, W. A.; Elsing, H. *J. Am. Chem. Soc.* **1994**, *116*, 5045. Measurements were made using a Finnegan MAT delta E dual inlet isotope ratio mass spectrometer. Reactions were performed in triplicate, and calculations were accomplished using the isotopic ratio in the *m*-nitrobenzyl alcohol product at partial reaction (R_p), in the residual substrate (R_s), and in the starting material (R_o). Equations 1 and 2 were used for calculation of the observed isotope effect for each trial in which the fraction of reaction, *f*, was determined by HPLC analysis as described above.

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

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

Isotopic composition of starting materials was determined by isotope ratio mass spectrometry or FAB mass spectrometry, and the isotope effect was corrected for incomplete isotopic incorporation. Isotope effects at the discriminating position were calculated using the remote label equations (O'Leary, M. H.; Marlier, J. F. *J. Am. Chem. Soc.* **1979**, *101*, 3300). Control experiments in which the isotopic ratio of pure *m*-nitrobenzyl alcohol was measured before and after isolation procedures ensured that no isotopic fractionation occurred during product recovery.

(12) The isotope effect of unity measured at the nitrogen atom of the nitro group of the benzyl ring is expected, since no electron delocalization can occur into the benzyl ring during phosphodiester bond cleavage to change the bond characteristics. The remote label therefore has no contribution to the primary or secondary isotope effects measured.

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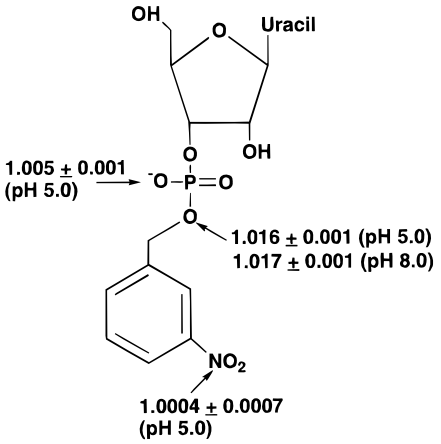

Figure 1. Isotope effects observed for the ribonuclease A catalyzed reaction.

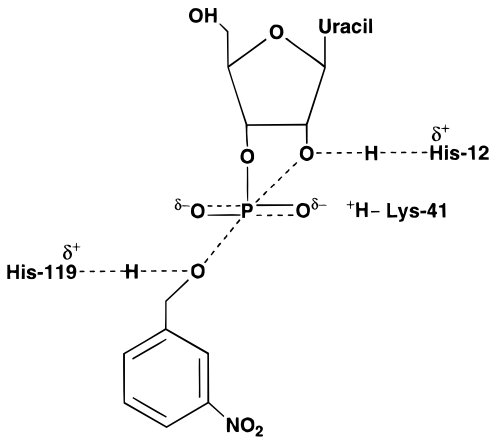

Figure 2. Proposed transition state structure for the ribonuclease A catalyzed reaction.

from the pH optimum (pH 8.0) demonstrates that a commitment is not present in this system. Thus, we conclude that chemistry is rate limiting under these conditions. Measurements of the other isotope effects were performed only at the pH optimum.

A normal primary isotope effect of 1.6% observed at the bridge oxygen atom indicates that there is considerable loss of bond order to the bridge oxygen atom in the transition state. This result is consistent with a concerted mechanism in which bond order is decreased to the leaving group in the transition state. However, the primary isotope effect by itself does not invalidate a two-step mechanism via a phosphorane intermediate. If the rate limiting step in such a mechanism were a breakdown of the phosphorane intermediate, a normal primary isotope effect would be expected.

However, evidence against a mechanism involving a phosphorane intermediate is provided by measurement of the nonbridge secondary isotope effect. A normal isotope effect of 0.5% was observed as the overall effect for both labeled atoms in the nonbridge position. A normal isotope effect at the nonbridge position indicates that there is some loss of bond order to the nonbridge oxygen atoms in the rate limiting transition state compared to the situation in the substrate before reaction. This is consistent with a concerted mechanism in which the transition state is slightly associative. In the case of a mechanism employing a phosphorane intermediate, the normal primary bridge isotope effect requires that the transition state under examination would involve the rate limiting breakdown of this intermediate. Calculations suggest that a dianionic phosphorane intermediate is unlikely due to energetic factors.[13] Nevertheless, if a dianionic phosphorane did form, it is likely that the transition state for breakdown of this unstable intermediate would be early (resembling the intermediate) and would reveal a large normal isotope effect (2.5−4%, see below) due to almost a complete loss of double bond character to the nonbridge oxygen atoms. Any hydrogen bonding stabilization would diminish by only a very small amount an observed isotope effect of this magnitude.

If a monoanionic phosphorane intermediate were formed, breakdown of this intermediate would have to involve deprotonation of a nonbridge oxygen in concert with protonation of the leaving group. In this case, this nonbridge oxygen atom would be at least partly protonated in the transition state. This would manifest itself as an inverse secondary isotope effect which would be as large as 1.5%[14] in the case of an early transition state and smaller in the case of a late transition state.[15] In any case, a normal isotope effect would not be expected. A precedent exists for this situation in the observation of an inverse nonbridge isotope effect of 1.6% for protonation of the phosphoryl group of the transition state for the snake venom phosphodiesterase catalyzed hydrolysis of 3,3-dimethylbutyl *p*-nitrophenyl phosphate.[16] It is possible that a small contribution from reaction coordinate motion could exist for deprotonation of the phosphorane. However, since it is the proton that is largely in motion and not the oxygen atom, the contribution of this effect would be small in comparison to the inverse isotope effect.

Thus the observed 0.5% normal secondary isotope effect is most consistent with the ribonuclease catalyzed cleavage of uridine 3′-*m*-nitrobenzyl phosphate proceeding via a concerted mechanism with a transition state having slightly associative character. We propose a transition state of the concerted reaction as shown in Figure 2. Herschlag has come to similar conclusions about ribonuclease catalysis based on his analysis of thio-effects.[17]

Assuming a constant bond order to phosphorus of 5, we have calculated that the maximum theoretical ^{18}O secondary isotope effect for complete conversion of a P═O double bond to a single bond is 4%,[18] while the largest experimental secondary isotope measured in solution is 2.5% for hydrolysis of *O*,*O*-diethyl *O*-(4-carbamoylphenyl) phosphate.[19] Using these values as limits, we calculate that between 0.13 and 0.20 bond order is lost to the nonbridge oxygen atoms in the transition state of the ribonuclease A catalyzed reaction. Therefore, the sum of the axial bond orders is 1.13−1.20 in the transition state, which is slightly associative. It is likely that Lys-41 acts to stabilize the increased negative charge formation on the nonbridge oxygen atoms.[20] The present data do not define the transition state for the cleavage of the 2′,3′ cyclic phosphate product; however, it is likely that ribonuclease A employs a similar mechanism to accomplish this step.

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