Isotope effect analyses provide evidence for an altered transition state for RNA 2'-O-transphosphorylation catalyzed by Zn^{2+}\(^{\dagger}\)

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Divalent ions are essential cofactors in the active sites of many phosphoryl transferases.\(^1\)–\(^6\) Although experimental information on how metal ions alter transition state (TS) structure is limited, the available data show that the effects can be quite large.\(^1\)–\(^2\) Non-enzymatic model reactions offer the potential to address basic questions about the roles of metal ions in biological catalysis.\(^1\),\(^2\),\(^7\)–\(^11\) Such information can be useful to help guide the design of artificial catalysts.\(^9\),\(^10\),\(^12\),\(^13\) RNA cleavage by 2'-O-transphosphorylation is a useful system to explore the roles of metal ions in phosphoryl transfer catalysis because this reaction is catalyzed non-enzymatically by divalent ions,\(^10\),\(^14\) organometallic compounds,\(^12\),\(^13\) as well as ribonucleases including ribozymes.\(^15\),\(^16\)

RNA 2'-O-transphosphorylation with displacement of 5'O and formation of a 2',3'-cyclic phosphate is catalyzed by both acids and bases and the mechanisms of these reactions are well studied.\(^8\),\(^11\),\(^17\) Thus, they provide contrasting examples for understanding RNA strand cleavage by divalent metals. Base catalysis involves equilibrium deprotonation of the 2'O nucleophile followed by nucleophilic attack. The mechanism is concerted via a late (product-like), anionic TS (Fig. 1A).\(^8\),\(^18\),\(^19\) Acid catalysis proceeds via a two-step mechanism involving the formation of a phosphorane intermediate in which one or both of the non-bridging oxygens may be protonated.\(^8\),\(^20\) Pseudorotation of the intermediate results in the formation of 2',5' isomerization products that are characteristic of the two-step mechanism of acid catalysis.\(^8\)

The catalysis of RNA transesterification by metal ions and organometallic compounds has also been the subject of intensive study because of the importance of divalent metal ion cofactors in enzymes and potential applications in synthetic catalysts.\(^9\),\(^10\),\(^12\),\(^14\),\(^21\) The pH dependences of the rate constants...
for RNA cleavage reactions catalyzed by metal ions are typically consistent with base catalysis. Often an apparent pKₐ consistent with the titration of metal coordinated water molecules is also observed. Increasing acidity of metal coordinated aquo ligands is generally correlated with a higher degree of rate enhancement. For displacement of basic alkyl groups like 5′O of ribose, catalysis by metal ions and metal ion complexes can result in a decrease in βKₐ reflecting a decrease in charge accumulation on the leaving group in the TS.²³⁻²⁶ Possible catalytic interactions involving divalent ions consistent with the available data include electrostatic stabilization of an anionic TS, inner sphere coordination of the nucleophile and leaving groups, and Brønsted acid/base catalysis involving coordinated water molecules¹⁻⁵ (Fig. 1B).

However, the precise modes of metal ion catalysis in both solution and enzyme reactions remain difficult to distinguish experimentally. This challenge is compounded now that recent biophysical and computational studies indicate that effects of metal ion catalysis on the TS structure depend on pKₐ of the leaving group, as well as the number and type of metal ions involved in catalysis.⁹,²¹ Kinetic isotope effect analyses can provide a valuable experimental method for distinguishing differences in ground state and transition state bonding.²³⁻³⁰ Such experimental data are critical for evaluating models of metal ion catalysis derived from computation. Therefore, we measured the ¹⁸O KIEs on the 2′O nucleophile (¹⁸Kₐ₂₃₅) and 5′O leaving group (¹⁸Kₐ₅₆) oxygens and the non-bridging phosphoryl oxygen (¹⁸Kₐ₈₉) as well as D₂O solvent effects for RNA 2′-O-transphosphorylations of uridylyl-3″-cUMP and guanosine (Fig. S1, ESI†) catalyzed by Zn²⁺ and by specific base.

Consistent with previous results,²² Zn²⁺ accelerates 2′-O-transphosphorylation of the dinucleotide UpG to yield uridine-2′,3′-cyclic-monophosphat (2′,3′-cUMP) and guanosine (Fig. S1, ESI†). The dependence of the observed rate constant on Zn²⁺ concentration shows saturation and suggests that two or more metal ions are involved in catalysis (Fig. S2, ESI†), although, a conclusive interpretation is complicated by the potential for changes in Zn²⁺ speciation at higher concentration. Accumulation of a 2′,3′ isomerization product is not observed, thus, a mechanism similar to acid catalysis involving the formation of a stable phosphorane is unlikely. Importantly, the log-linear dependence of the rate constant for Zn²⁺ catalysis on pH is consistent with base catalysis. Often an apparent value of 0.14. The blue dashed line a simulation using eqn S3 (ESI†) for a model involving acid catalysis in which there is a modest inverse fractionation factor (φ²⁻¹ = 2) due to an increase in the protonated form of the catalyst at constant pL that necessitates a large offsetting normal fractionation factor (φ² = 0.05). The solid black line assumes two normal fractionation factors: one reflecting the change in pKₐ of the 2′OL (1/φ²⁻¹ = 0.2) and a second normal contribution of φ²⁻¹ = 0.4.

To gain information on whether Zn²⁺ catalysis alters the transition state by transfer of protons in the TS, we employed proton inventory analysis.³¹⁻³² This approach measures the dependence of the observed rate constant on the fraction D₂O in reactions containing mixtures of H₂O and D₂O. These data may be used to evaluate alternative models for the number of exchangeable protons that contribute to the observed SKIE and estimate the magnitude of the effect from each site (φ values). Both Zn²⁺ catalysis and specific base catalysis show similar, large normal SKIES (³¹Kₐ₁ = 7.7 ± 0.9 and ³¹Kₐ₁ = 13.2 ± 0.5). A linear model for one titratable group affected by H/D substitution can fit the data (Fig. 2, red line), however, nonlinear residuals make this model unlikely. Models for Zn²⁺ transition state stabilization have proposed general acid catalysis involving a metal coordinated water or hydronium ion.²¹,²² In this mechanism ΔpKₐ of +0.85 in D₂O for the metal coordinated water would increase the concentration of the active form of the catalyst. This effect in turn would impart an inverse (kₖₒₙ₁/kₖₒ₈₁ < 1) value of φ² of ca. 0.14. That is, the reaction would be 7-fold faster in D₂O compared to H₂O. Such a large inverse effect would have to be more than offset by the presence of large normal fractionation factors in order to result in the >10-fold slower rate constant that is experimentally observed in D₂O. The presence of both normal and inverse φ values would result in an arch-shaped proton inventory, which is not observed experimentally (Fig. 2, blue line).

The proton inventories for both reactions are consistent with two normal fractionation factors: a large equilibrium effect due to differences in 2′O solvation in the ground state (1/φ²⁻¹ ≈ 0.2) and a second normal contribution of lower magnitude (φ²⁻¹ ~ 0.4) observed in previous SKIE analyses of RNA cleavage (Fig. 2, black line), attributed to differences in TS solvation. Proton inventory analyses, however, are known to have limited ability to distinguish models involving more than two exchangeable protons.³¹,³² Nonetheless, a simple interpretation is that there is little change in the number or contribution of catalytic modes involving proton transfer in the presence of the Zn²⁺ catalyst.
To better understand the effects of metal ion catalysis on O–P bonding, we measured 18O KIEs for Zn2+ catalyzed RNA transesterification. Heavy atom 18O KIEs arise due to differences in the vibrational modes in the ground state and transition state. For measurement of 18kNuc, 18kLG and 18kNPO, the appropriate sites specifically enriched with UpG molecules were synthesized and the KIEs were measured by internal competition18,33,34 (Fig. 3A). Previous KIE analyses on RNA and other phosphodiesters (see Table S1, ESI†) provide a context for interpreting the current results in terms of a general TS structure.

A large normal value of 18kLG of 1.034(3) (standard errors in the last digit are shown in parenthesis) that is observed for specific base catalysis is attributable to an advanced 5’O–P bond cleavage.18,20,33,35,36 A similarly large 18kLG of 1.0272(1) is observed for base catalyzed transesterification of U-3’-m-nitrobenzylphosphate, which has a similarly unreactive leaving group (pKₐ ca. 13.4). Diester reactions with good leaving groups (e.g. nitrophenol, pKₐ 7) react via early transition states with 18kLG values near unity.37,38 However, 18kLG for the Zn2+-catalyzed reaction is significantly less [1.015(2)] than the specific base reaction demonstrating a stiffer 5’O bonding environment in the TS due to metal ion catalysis.

The observed inverse 18kNuc value of 0.997(1) for the specific base reaction also reflects a late TS, and results from a large inverse contribution of 0.980 due to formation of the 2’O–P bond.18,19 However, this contribution is partially offset by the large normal equilibrium isotope effect of ca. 1.024 due to loss of the 2’O–H stretching mode.18,36,39 In contrast, normal 18kNuc values (1.02–1.04) are observed for reactions with early TSs in which nucleophilic attack is rate limiting (Table S1, ESI†).19,38 Thus, the observed inverse 18kNuc for the Zn2+-catalyzed reaction is also consistent with a late TS.

The secondary 18O effects on the non-bridging oxygens are near unity for both the specific base and metal ion catalyzed reactions. This result is consistent with both reactions proceeding by similar product-like, anionic TSs.20,35 For comparison, an inverse 18kNPO of 0.9904 is observed for acid catalysis of U-3’-m-nitrobenzylphosphate transesterification and 0.991(1) for RNA (Table S1, ESI†), both of which are proposed to proceed via a stable phosphorane.26,37 Therefore, this mechanism is unlikely for Zn2+ catalysis. Formation of new vibrational modes give rise to normal equilibrium isotope effects on water coordination by metal ions.40,41 However, Mg2+ coordination to ATP was observed to result in an 18O isotope effect no larger than 1.001.42 A simple interpretation is that the non-bridging oxygen bonding environment is unchanged in the metal catalyzed reaction. However, the potential for multiple contributions to the observed 18kNPO effect that could be offsetting or complex obscures a simple interpretation.

The interpretations of KIE data with respect to the TS structure are aided by DFT calculations examining the effect of different numbers of ions and different Zn2+ binding modes on the TS structure. Recently, Chen et al. described a set of alternative Zn2+ binding modes that were analyzed with respect to their effects on the predicted KIE values.43 One or two metal ion interactions with the non-bridge phosphoryl oxygen or nucleophile involving either direct coordination or interaction via coordinated water alone either gave no significant difference in the observed KIEs or resulted in an early TS inconsistent with experimental data. A two metal ion mechanism is found to result in calculated KIEs that are consistent with the observed values reported here (Fig. 3C). In this model, the first Zn2+ ion (M_A) binds to the NPOs while the second ion, M_B,
coordinates to the 5′O leaving group. $M_A$ stabilizes accumulation of negative charge on the non-bridging oxygens allowing the formation of a TS that is more associative. Like the TS for specific base catalysis,$^{18,33,36}$ the TS for Zn$^{2+}$ catalysis involves advanced $2′$-P bonding, and this effect together with the $M_jOH_2$ mediated H-bond results in an observed $^{18}\Delta_{LOC}$ that is overall inverse. H-bonding between the metal coordinated water and the nucleophile was proposed, however, $2′$-P bonding in the model is advanced and the $2′$ O acts only as an H-bond acceptor. Thus, only a small contribution to the observed SKIE would be expected.$^{31,32}$ It may be inferred, however, that due to its proximity $M_B$ may be pre-positioned to act as a specific base and facilitate transfer of the proton in a pre-equilibrium step.

Cleavage of a series of uridine-3-alkylphosphates by Zn$^{2+}$ ions under the same reaction conditions used here for KIE measurements results in a significantly lower $\beta_{LG}$ (−0.43 to −0.32)$^{22}$ compared to −1.28 reported for specific base catalysis.$^{44}$ The decrease in bond cleavage reflected in the shorter 5′O-P bond lengths in the specific base and Zn$^{2+}$-catalyzed models (2.17 Å) (Fig. 3C) is consistent with the observed difference in charge accumulation indicated by the LFER results. In contrast to the late TS for RNA transphosphorylation, KIE, LFER data and computational results indicate that the uncatalyzed cyclization of 2-(hydroxpropyl)-4-nitrophenyl phosphate (HPPNP) and similar RNA models with activated leaving groups occur via early TSs. For these reactions there is little phosphorus–oxygen bond fission to the leaving group and minimal nucleophilic bond formation in the TS.$^{18,20,37,38}$ Catalysis of HPPNP transphosphorylation by a dinuclear Zn$^{2+}$ compound compared to a specific base results in a larger $^{18}\Delta_{LG}$ (1.0113(5) versus 1.0064(9)) and a smaller $^{18}\Delta_{LOC}$ (1.0116(10) versus 1.0327(8)) (Table S1, ESI†). The change in the magnitude of these effects reflects an overall later TS with greater nucleophilic bond formation.$^{35}$ Recent computational simulations of HPPNP transphosphorylation are consistent with the KIE data and suggest a more associative TS,$^{46}$ although one that is still early compared to the TS for RNA transphosphorylation demonstrated here. Nonetheless, a similar theme is observed relevant to enzyme mechanism. Transphosphorylation catalyzed by Zn$^{2+}$ and Zn$^{2+}$ complexes is accompanied by selection of an altered TS arising from the preferential stabilization of negative charge.

Notes and references