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Review

Heavy atom labeled nucleotides for measurement of kinetic isotope effects☆

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ABSTRACT

Experimental analysis of kinetic isotope effects represents an extremely powerful approach for gaining information about the transition state structure of complex reactions not available through other methodologies. The implementation of this approach to the study of nucleic acid chemistry requires the synthesis of nucleobases and nucleotides enriched for heavy isotopes at specific positions. In this review, we highlight current approaches to the synthesis of nucleic acids enriched site specifically for heavy oxygen and nitrogen and their application in heavy atom isotope effect studies. This article is part of a special issue titled: Enzyme Transition States from Theory and Experiment.

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1. Introduction

Kinetic isotope effects (KIEs) offer an extremely powerful method for interrogating enzymatic transition states and active sites. The substitution of a single atom represents the smallest possible perturbation to a chemical system, and replacement of an atom with a heavy isotope reports directly on changes to the bonding environment of that atom in the rate-limiting step of the reaction [1]. While this approach has been used frequently in the interrogation of many enzymatic reactions [2–5], the use of heavy atom isotope effects to measure enzymatic reactions involving nucleotides has been limited by several technical challenges.

First, the difference in mass between isotopes of heavy atoms is small, so detecting primary isotope effects for oxygen and nitrogen requires a highly precise measurement of relative rates for isotopologues. This challenge can be met by determination of relative rates through internal competition rather than direct rate measurements [6] and by application of highly precise mass spectrometric methods [7]. Second, multi-step processes that involve reaction steps prior to chemistry can reduce the magnitude of observed isotope effects [8,9]. This problem is especially pronounced in RNA catalysis where pre-chemical processes like folding or strand annealing can be rate-limiting or occur with rate constants on the same order as k_{cat}. Therefore, analysis of isotope effects requires a basic kinetic understanding of a given reaction, and potentially the development of an alternative system with rate-limiting chemistry. Additionally, the interpretation of isotope effects can be complicated by a lack of knowledge of the starting ground state of the reaction and the influence of chemical entities not explicitly involved in bond formation like metal ions. Measured isotope effects can be interpreted with greater clarity when compared to measured isotope effects for reference reactions [10] and complemented with computational studies. Continued improvements in multiscale modeling of complex biochemical systems has greatly aided in the interpretation of measured isotope effects. Finally, measurement isotope effects nucleic acid chemistry requires the synthesis of nucleotides site-specifically enriched for heavy isotopes. While the literature is rife with strategies for the isotopic enrichment of nucleotides, very few are site specific with respect to atomic position as most of these methodologies reported to date are for NMR studies [11–13].

Meeting these challenges enables the use of KIE analysis on reactions could not have been probed previously, which has the potential to greatly enhance and deepen our understanding of these processes. For example, in more than three decades of studies on RNA catalysis, our understanding of transition state interactions is well developed [14];

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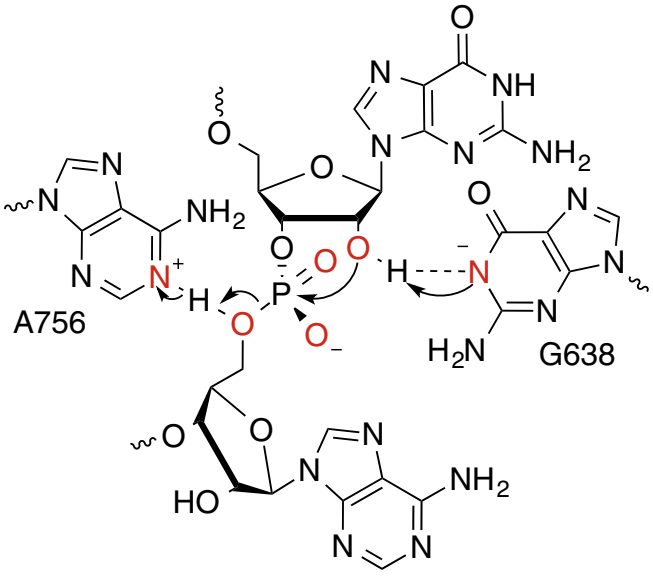
however, our understanding of transition state structure remains in its infancy. Herein, we highlight a number of experiments that have employed nucleotides enriched site specifically with heavy atoms for isotope effect measurements and describe the synthetic challenges involved in producing these compounds. Our motivation in this area stems from a long-standing interest in RNA catalysis and the fact that there has been no heavy atom isotope effect analysis for any nucleolytic ribozyme. Therefore, we frame this review around the measurements required to probe the transesterification reaction catalyzed by several small endonucleolytic ribozymes. In doing so, we hope to illustrate the complexity and subtlety involved in these studies and to motivate the need for a variety of site-specifically labeled nucleotides.

Most nucleolytic ribozymes catalyze phosphoryl transfer via an S_N2-like mechanism, in which a 2′ hydroxyl nucleophilically attacks its own 3′ phosphodiester causing strand scission, displacing a 5′ hydroxyl leaving group and producing a 2′, 3′ – cyclic phosphate (Scheme 1). The isotope effect analysis of this process would require, at a minimum, heavy atom enrichment of the 2′ hydroxyl (to determine the extent of nucleophilic attack) and 5′ hydroxyl (to determine the extent of leaving group departure). However, the interpretation of these isotope effects would be complicated by the fact that the oxygen atoms are involved in both bond cleavage and bond formation over the course of the reaction. The 5′ oxygen, for example, loses its bond to the scissile phosphate and becomes protonated spontaneously. If strand scission and protonation both occur to some extent in the transition state, then it would be difficult to determine the relative contribution of each process to the measured isotope effect without additional information. Therefore, the measurement of isotope effects for atoms implicated in proton transfer *to and from* the leaving group and nucleophile, respectively, greatly simplifies the interpretation of isotope effects for the nucleophile and leaving group.

In several nucleolytic ribozymes, including the hepatitis delta virus (HDV) [15], Varkud satellite (VS) [16], Hairpin [17] ribozymes, and potentially the recently discovered Twister ribozyme [18], proton transfer is facilitated by one or two nucleobases positioned in the active site. For example, in the VS ribozyme, adenine and guanine nucleobases positioned in the active site have been implicated in general acid and general base catalysis, respectively, whereby the N1 atom of the catalytic guanine abstracts a proton from the 2′ hydroxyl nucleophile while the N1 atom of the catalytic adenine donates a proton to the 5′ hydroxyl leaving group [16]. The measurement of isotope effects for these two nitrogen atoms would serve two purposes. First, such measurements could further implicate the involvement of the putative general acid and general base in proton transfer during the rate-limiting step. Second, coupled with measurements of isotope effects for the 2′ hydroxyl nucleophile and 5′ oxygen leaving group, they could help to clarify the extent of nucleophilic attack and leaving group departure in the transition state.

In addition to the aforementioned isotope effects, the measurement of a number of secondary isotope effects would also be helpful in elucidating the precise mechanism employed by nucleolytic ribozymes. Of particular interest are the non-bridging phosphoryl oxygen atoms. While they are not directly involved in bond formation or cleavage, the measurement of isotope effects for non-bridging oxygen atoms provides information on the structure of the transition state by reporting on the bonding environment around the scissile phosphate. Additionally, the interrogation of deuterium or tritium isotope effects for the hydrogen atoms attached to the 2′, 3′ and 5′ carbon atoms proximal to the scissile phosphate could provide information about changes to the bonding environment around those carbons.

In summary, an exhaustive interrogation of the mechanism of nucleolytic ribozyme with isotope effects would require (1) site-specific enrichment of heavy atoms at the 2′ and 5′ oxygen; for ribozymes outside of class II, like the group I intron, heavy atom at the 3′ oxygen position would also be required; (2) the non-bridging phosphoryl oxygen; (3) the 2′, 3′, and 5′ hydrogen; and (4) any nucleobase atom with suspected involvement in proton transfer—generally this would include N1 nitrogen for purines and N3 nitrogen for pyrimidines. In this review, we focus primarily on methods for the synthesis of nucleotides with heavy heteroatoms since, unlike carbon and hydrogen, these atoms are directly involved in bond formation and scission and thus likely to exhibit primary isotope effects. Moreover, we highlight only state-of-the-art syntheses for these substrates with an emphasis on methodologies that generate substrates isotopically enriched at a single atomic position. Interested readers should also consult excellent reviews by Theodorou et al. [19] and Milecki [20], which contain a more complete account of isotopically labeled nucleotides.

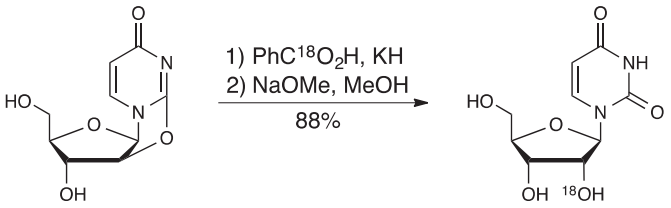


Scheme 1. Putative mechanism nucleolytic cleavage by VS ribozyme [16]. Heavy atoms for which measured isotope effects would aid in the determination of the transition state structure are highlighted in red.

2. Labeling sugar oxygen

Uridine containing 2′-^{18}O has been synthesized by heating 2,2′-cyclouridine in the presence of labeled benzoic acid, potassium hydride in dimethylformamide to give [2′-^{18}O]benzoyluridine followed by treatment with methoxide to convert the benzoylated product into uridine [21] (Scheme 2). The process is quite straightforward requiring only two steps with 88% yield and employs a relatively inexpensive label source, benzoic acid; however, because it utilizes a cyclic derivative of uridine as a starting material, it may be incompatible with the synthesis of other nucleosides labeled at the 2′-O position. However, synthesis of 2′-O-labeled cytidine could be indirectly achieved through the known conversion of uridine to cytidine by activation at the C4 position with tetrazole followed by ammonolysis [22].

[3′-^{18}O]Uridine has also been prepared by a related method that begins with a cyclic uridine derivative [23] (Scheme 3). Labeled benzoyl chloride was reacted with 2,3′-cyclouridine at the 2′ position in the presence of pyridine to give 2′-^{18}O-benzoyl-2,3′-cyclouridine, which underwent thermal Fox rearrangement revealing [2′-^{18}O]2,2′-



Scheme 2. Conversion of 2,2′-cyclouridine to [2′-^{18}O]uridine [19].

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