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A Method to Determine ^{18}O Kinetic Isotope Effects in the Hydrolysis of Nucleotide Triphosphates

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Abstract

A method to determine ^{18}O kinetic isotope effects (KIE) in the hydrolysis of GTP is described that is generally applicable to reactions involving other nucleotide triphosphates. Internal competition, wherein the substrate of the reaction is a mixture of ^{18}O -labeled and unlabeled nucleotides, is employed and the change in relative abundance of the two species in the course of the reaction is used to calculate KIE. The nucleotide labeled with ^{18}O at sites of mechanistic interest also contains ^{13}C at all carbon positions, while the ^{16}O -nucleotide is depleted of ^{13}C . The relative abundance of the labeled and unlabeled substrates or products is reflected in the carbon isotope ratio ($^{13}\text{C}/^{12}\text{C}$) in GTP or GDP, which is determined by use of a liquid chromatography-coupled isotope ratio mass spectrometer (LC-coupled IRMS). The LC is coupled to the IRMS by an IsolinkTM interface (ThermoFinnigan). Carbon isotope ratios can be determined with accuracy and precision greater than 0.04%, and are consistent over an order of magnitude in sample amount. KIE values for Ras/NF1³³³-catalyzed hydrolysis of [$\beta^{18}\text{O}_3$, ^{13}C]GTP were determined by change in the isotope ratio of GTP or GDP or the ratio of the isotope ratio of GDP to that of GTP. KIE values computed in the three ways agree within 0.1%, although the method using the ratio of isotope ratios of GDP and GTP gives superior precision (< 0.1%). A single KIE measurement can be conducted in 25 minutes with less than 5 μg nucleotide reaction product.

Keywords

Ras; GTPase Activating Protein (GAP); GTP hydrolysis; Transition state; Enzyme mechanism; Kinetic isotope effect (KIE); Isotope ratio mass spectrometry (IRMS); Chemical reaction interface (CRI)

Introduction

Phosphoryl transfer reactions involving ATP and GTP constitute arguably the most ubiquitous biological reactions. The utility of such reactions in biology may be attributed to the fact that cleavage of a phosphoanhydride bond is highly exothermic[1,2], while the kinetic barrier to bond cleavage is high[3]. Many enzymes facilitate phosphoanhydride bond cleavage and utilize

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the released free energy to accomplish various tasks in the cell[4]. These include, but are not limited to, motor proteins, molecular pumps, chaperones, and many enzymes in metabolic pathways that couple ATP hydrolysis to otherwise thermodynamically unfavorable reactions. G proteins[5] or protein kinases[6] use phosphoryl transfer as a means to modulate their own activity or that of their target.

One of the central tenets of enzymology is that enzymes achieve catalysis by preferentially stabilizing the transition state relative to the ground state[7-9]. Therefore, knowledge of the transition state structure is key to understanding the catalytic mechanism of an enzyme. Observation of kinetic isotope effects (KIE) form the basis for one of the most powerful methods to study the transition state[10]. KIE refers to the effect of isotopic substitution on reaction rate and is mathematically defined as the ratio of the reaction rate for the unlabeled reactant or substrate to that of the labeled reactant or substrate. The KIE observed for a non-catalyzed reaction is due only to differences in the vibrational properties of the transition state and the ground state, and therefore represents the “intrinsic KIE” associated with that reaction. However, the KIE observed for an enzyme-catalyzed reaction is a function of both the intrinsic KIE in the chemical step and the rate constants for non-chemical steps such as substrate binding and release, formation of intermediates, and conformational changes [11]. Frequently, the observed KIE in an enzymatic reaction is scaled down, relative to the intrinsic KIE, by forward and reverse commitment factors that depend on the extent to which the chemical step is rate-limiting. In cases where chemistry is completely or almost completely rate-limiting, the defining features of the transition state, such as bond lengths and bond angles, can be deduced from the observed KIEs for atomic positions that undergo changes in these properties at the transition state[12,13].

KIEs can be measured by direct comparison of reaction rates, equilibrium perturbation, or internal competition methods [14]. Direct comparison of reaction rates that are measured separately for the unlabeled and labeled substrates is sufficiently precise if the isotope effect is large (> 1.1). Equilibrium perturbation is limited to reactions that are reversible and can be monitored with a spectrometer [15]. If the isotope effect is small (< 1.1), as is the case for ^{18}O KIEs, internal competition is the method of choice. In this approach both isotopically labeled and unlabeled substrates are present in the reaction mixture, and the change in their relative abundance is used to calculate the KIE. The accuracy and precision of the KIE determination depends primarily on that of the measurement of relative abundance. In many KIE studies, measurement of the relative abundance is carried out by use of an isotope ratio mass spectrometer (IRMS), which is a specially configured instrument for precise measurement of isotopic abundance in gaseous molecules, e.g., the ratio of ^{13}C and ^{12}C in CO_2 . Therefore a critical aspect of the experimental design is the method used to convert the isotopically labeled sites of the substrate or product into a gaseous product such that the relative abundance of the labeled and unlabeled species of the reactants or products is reflected in the isotope ratio of the gas derivative. In cases where it is not experimentally tractable to do so, a method involving substrate labeled at both the site of chemical interest and at a remote “reporter” site may be used[16]. The isotopic abundance at the reporter site is monitored to yield the relative abundance of the doubly labeled and unlabeled substrates or products.

In KIE studies of phosphoryl transfer reactions, the reaction mixture contains substrate that is isotopically enriched with ^{18}O at one or more positions and substrate in which ^{18}O is at natural abundance (0.2%). Direct measurement of oxygen isotope ratios in reactant or products, or oxygen species derived from them, presents formidable experimental challenges. Cleland and colleagues used a double labeling method to determine ^{18}O KIE in hexokinase-catalyzed phosphoryl transfer from ATP using the exocyclic amino group of adenine as the “reporter” site [17]. However, there has been no further study using this technique, presumably because of the difficulties associated with analytical chemistry: the method requires a large quantity of

ATP (40 mg or 80 μ mol ATP per experiment) and isolation and conversion of the ATP amine group to N_2 in preparation for IRMS requires chemical manipulation. We are not aware of any ^{18}O KIE study on phosphoryl transfer reactions involving nucleotides other than ATP.

We have developed a double labeling, internal competition method to determine ^{18}O KIEs in reactions involving nucleotide triphosphates. The substrate mixture is composed of ^{13}C -depleted nucleotide and nucleotide that is labeled with ^{18}O at sites of mechanistic interest and is also uniformly enriched with ^{13}C . The relative abundance of the labeled and unlabeled substrates or products is thereby reflected by the macroscopic carbon isotope ratio ($^{13}C/^{12}C$). Another critical aspect of the method is the use of a liquid chromatography-coupled isotope ratio mass spectrometer (LC-coupled IRMS) in the determination of the carbon isotope ratio of the nucleotide. Connecting the LC and the IRMS is an IsolinkTM interface (ThermoFinnigan) that chemically oxidizes carbon in the sample into CO_2 in preparation for IRMS[18]. The use of LC/oxidization/IRMS allows direct determination of the isotope ratios of product and substrate, and thus the high sensitivity of the procedure. A KIE can be determined in a 25 minute chromatographic run with a precision around 0.1% using only 5 μ g (10 nmole) GTP as substrate. We describe this method of KIE determination here and demonstrate its sensitivity and precision. As an example, we present individual determinations of KIE for hydrolysis of [$\beta^{18}O_3$, ^{13}C]GTP, which is ^{18}O -labeled at the β - γ bridge and the two β nonbridge oxygen positions, catalyzed by Ras and NF1³³³.

Materials and Methods

Materials

^{13}C -labeled GMP and GTP (ammonium salt) were purchased from Spectra Gases, Inc. Enrichment in ^{13}C is greater than 99% as determined by electrospray mass spectrometry. $H_2^{18}O$ (97% enriched) was from Isotech. Carbamate kinase (*Enterococcus faecalis*), guanylate kinase (porcine brain), pyruvate kinase (rabbit muscle), glycerokinase (*E. coli*), phosphoenolpyruvate (sodium), carbamoyl phosphate (sodium), cyanate (potassium), ADP (sodium), and phosphorus pentachloride were from Sigma. Nuclease P1 (*Penicillium citrinum*) was from Wako International, Japan. The reagents used for LC and Isolink, sodium peroxodisulfate, H_3PO_4 , KH_2PO_4 , and silver nitrate, were from Fluka. HPLC grade water was from Sigma. Guanine nucleotide kinase (*Escherichia coli*) was subcloned into a pET28 vector, over-expressed in *E. coli*, and purified by Ni affinity chromatography. $G\alpha_{i1}$ (Rat) was prepared as described earlier [19].

Synthesis of [$\beta^{18}O_3$, ^{13}C]GTP

[$^{18}O_4$]P_i was synthesized as tetrabutylammonium salt from PCl_5 and $H_2^{18}O$ as described [20]. Carbamoyl [$^{18}O_4$]phosphate was synthesized by incubating a 690 μ l mixture containing 157 mM [$^{18}O_4$]P_i, 739 mM KCNO, and 492 mM sodium acetate, pH 4.9, at 37°C for 20 minutes. The solution was then mixed with a 2.6 ml mixture containing 20 mg [^{13}C]GMP, 0.2 mg ADP, 9 units guanylate kinase, 50 units of carbamate kinase, 15 mM $MgCl_2$, and 75 mM HEPES, pH 7.5. The reaction mixture was incubated at 37°C for 4 hours. The product of the preceding coupled reactions, [$\beta^{18}O_3$, β - $\gamma^{18}O$, $\gamma^{18}O_3$, ^{13}C]GTP, was then purified by anion exchange chromatography on a HiTrap Q column (5 ml, GE Healthcare). Elution was accomplished by a 20 column volume, 0 – 1 M gradient of ammonium acetate, pH 7.1. [$\beta^{18}O_3$, β - $\gamma^{18}O$, $\gamma^{18}O_3$, ^{13}C]GTP came off the column at approximately 0.7 M ammonium acetate, which was removed by lyophilization afterwards. [$\beta^{18}O_3$, β - $\gamma^{18}O$, $\gamma^{18}O_3$, ^{13}C]GTP was then converted to [$\beta^{18}O_3$, ^{13}C]GDP in a 1 ml reaction volume containing 1 mg $G\alpha_{i1}$, 1 mM $MgCl_2$, 200 mM ammonium sulfate, and 20 mM Tris-HCl, pH 7.5 after 15 hour incubation at 25°C. [$\beta^{18}O_3$, ^{13}C]GDP was purified by ion exchange column chromatography and phosphorylated with unlabeled carbamoyl phosphate by carbamate kinase to yield

[$\beta^{18}\text{O}_3$, ^{13}C]GTP [21]. The enrichment level of ^{18}O is about 96% as determined by electrospray mass spectrometry.

Synthesis of [^{12}C]GTP

^{13}C -depleted nucleotide monophosphates (NMP) were produced in *E. coli* grown on ^{12}C glucose, and purified as described [22]. GMP was phosphorylated to GTP in coupled reactions catalyzed by pyruvate kinase and *E. coli* guanine nucleotide kinase, which catalyzes the phosphorylation of GMP with high specificity [23,24]. The phosphorylation reaction was carried out in a 4 ml reaction volume containing 80 mg nucleotide monophosphates (about 20 mg of each nucleotide), 2 mg ATP (ammonium salt), 60 mg phosphoenolpyruvate, 30 units pyruvate kinase, 5 mg guanine nucleotide kinase, 5 mM MgCl_2 , 100 mM KCl, and 50 mM Tris-HCl, pH 8.0. The reaction was complete in 20 minutes at 25°C. [^{12}C]GTP was separated from other nucleotides by anion exchange chromatography on two 5 ml HiTrap Q columns in tandem. NMPs were removed in this step, but residual [^{12}C]ATP remains. The contaminating [^{12}C]ATP was converted by glycerokinase to [^{12}C]ADP in a 4 ml reaction volume containing 20 mg [^{12}C]GTP, 5 units glycerokinase, 3.4 mM Glycerol, 2 mM MgCl_2 , and 100 mM Glycine-KOH, pH 10. The hydrolysis was complete after 1 hour incubation at 22°C. Anion exchange column chromatography was repeated to remove [^{12}C]ADP. Remaining impurities were removed with a C18 column (300 $\times$ 19 mm ID, 15 μm , 300 Å, Delta Pack, Waters). [^{12}C]GTP was loaded and eluted isocratically with a buffer containing 1.5% Methanol and 200 mM triethylammonium bicarbonate, pH 7.5. Finally, [^{12}C]GTP eluted from the C18 column was loaded onto a HiTrap Q column, eluted by a gradient of ammonium acetate, lyophilized, and stored at -20°C .

Preparation of substrate mixture of ^{12}C and ^{13}C nucleotide

The precision of isotope ratio measurements using an IRMS is optimal when the isotope ratio of the analyte is close to natural abundance. Accordingly, ^{13}C -labeled nucleotide and ^{13}C -depleted nucleotide were mixed in proportion to yield an apparent carbon isotope ratio of 1.11 $\pm$ 0.04 %. The substrate mixture was further polished by ion-exchange column chromatography on a HiTrap Q column with ammonium acetate as eluting salt. The substrate mixture was then lyophilized, re-dissolved in water to a concentration of 2 mM, and stored at -20°C .

Protein expression and purification

A synthetic gene encoding residues 1 to 166 of human H-Ras [25] was sub-cloned into a pET28 vector. pGEX2T-CDC25 [26] and pGEX4T3-NF1³³³ [27], which encode the CDC25 domain of mouse CDC25 and the catalytic fragment of human neurofibromin 1 (NF1), respectively, as N-terminal fusion proteins with glutathione-S transferase (GST), were provided by Dr. Wittinghofer. Ras and CDC25 were co-expressed in *E. coli* strain BL21(DE3) and co-purified as a complex by Ni-affinity, glutathione affinity, and ion-exchange column chromatography. NF1³³³ was expressed in BL21(DE3) and purified by glutathione affinity and ion-exchange chromatography (HiTrap Q).

Steady state hydrolysis of GTP catalyzed by Ras and NF1³³³

A typical GTP hydrolysis reaction catalyzed by Ras and NF1³³³ was carried out in 30 μl volume that contained 4.0 mg/ml Ras-CDC25 complex, 6 $\mu\text{g/ml}$ NF1³³³, 1 mM GTP, 25 μM MgCl_2 , and 50 mM HEPES, pH 7.5 at 22°C. Typically a reaction reached 50% completion in 50 minutes. At different time points in the course of the reaction, aliquots of 10 μl were taken from the reaction mixture. Proteins in the sample were denatured in a 100°C heating block for 20 seconds and removed by centrifugation. The sample was diluted with H_2O to 30 μl and stored at -20°C .

Measurement of peak areas and isotope ratios of GDP and GTP

The isotope ratios and peak areas of GDP and GTP were determined on an isotope ratio mass spectrometer (Finnigan MAT252) that was coupled to a HPLC system (Finnigan Surveyor) through an Isolink interface (Thermo Electron). A schematic diagram of the Isolink interface is shown in Figure 1. In short, the Isolink converts carbon in HPLC effluents to CO₂, extracts the CO₂ from the liquid phase, dries it, and introduces it into the IRMS [18]. A sample prepared as described in the previous section was loaded into a 20 µl injection loop and injected onto a high pressure DEAE column (BioBasic AX, Thermo Electron) in the presence of 100% buffer A (5 mM KH₂PO₄, pH5.3). GDP and GTP in the sample were separated with an elution program that consisted of a 4 minute step of 0% B (750 mM KH₂PO₄, pH5.3), a 3.5 minute step of 15% B, an 8 minute gradient to 70% B, and a 4 minute gradient to 100% B at a flow rate of 400µl/min. Inside the Isolink, HPLC effluent was mixed through a T joint with a solution of sodium peroxodisulfate (1M) and a solution of phosphoric acid (1.5M) with a catalytic amount of silver nitrate (0.5 mM) each at 50µl/min. The solutions for the HPLC and the Isolink interface were prepared using HPLC grade water and degassed under vacuum in an ultrasonic bath before use. Carbon in the solution was oxidized quantitatively into CO₂ in a heated oxidation reactor, which was maintained at 99.9°C. CO₂ escaped from the aqueous phase through thin membranes into a counter flow of helium in the separation unit. The helium stream carrying CO₂ was subsequently dried by two Nafion units in series and admitted into IRMS. Signals corresponding to mass 44 (¹²C¹⁶O¹⁶O), 45 (¹³C¹⁶O¹⁶O and ¹²C¹⁷O¹⁶O), and 46 (¹²C¹⁸O¹⁶O, ¹³C¹⁷O¹⁶O, and ¹²C¹⁷O¹⁷O) were monitored by IRMS in separate channels. Chromatographic peaks were defined by a starting slope of 0.2 mV/S and an ending slope of 0.4 mV/S on mass 44 curve. Peaks of GDP and GTP were integrated, yielding both peak areas and carbon isotope ratios according to standard procedures implemented in the Isodat 2.0 software.

Computation of KIEs

Three alternative methods (equations 1-3) were used to compute KIE, where R_s or R_p is the relative abundance of the doubly labeled nucleotide and ¹³C-depleted nucleotide in the substrate (R_s) or the product (R_p), respectively, at a fractional reaction extent f , and R_0 or R_{100} is the relative abundance in the starting substrate or the product after complete hydrolysis, respectively [14]. Since ¹³C was not completely depleted in the ¹³C-depleted nucleotide, the observed carbon isotope ratios in GTP (R_{s_obs}) or GDP (R_{p_obs}) were corrected to obtain the change in the relative abundance. For example, R_p/R_s was computed according to Equation (4), where R_{res} is the residual carbon isotope ratio (0.09) in the ¹³C-depleted nucleotide as determined by LC/oxidization/IRMS. R_s/R_0 and R_p/R_{100} were calculated similarly. Fractional reaction extent was calculated from mass 44 peak areas of GDP and GTP according to Equation (5).

$$KIE_s = \frac{\ln(1-f)}{\ln[(1-f)R_s/R_0]} \quad (1)$$

$$KIE_{p/s} = \frac{\ln(1-f)}{\ln \frac{1-f}{1-f+R_p/R_s}} \quad (2)$$

$$KIE_p = \frac{\ln(1-f)}{\ln(1-fR_p/R_{100})} \quad (3)$$

$$\frac{R_p}{R_s} = \frac{R_{p_obs} - R_{res}}{R_{s_obs} - R_{res}} \quad (4)$$

$$f = \frac{Area(GDP)}{Area(GDP) + Area(GTP)} \quad (5)$$

Results and Discussion

Analytical scheme

We employed an internal competition method using ^{18}O , ^{13}C -labeled GTP and ^{13}C -depleted GTP. The relative abundance of the labeled and unlabeled substrates or products is reflected in the carbon isotope ratio of GTP or GDP and determined using a LC/oxidization/IRMS system. Reaction mixtures containing GDP and GTP were injected directly unto the LC/oxidization/IRMS after removal of proteins by brief heating and centrifugation. GDP and GTP were separated on an anion exchange column, converted into CO_2 in the Isolink interface, and introduced into the IRMS. Peak areas and isotope ratios of GDP and GTP furnished by IRMS were used to compute KIE according to equations (1-5).

Accuracy and precision of LC/oxidization/IRMS

Accurate determination of KIE depends on the accuracy with which changes in isotope ratios are measured. Because of the lack of GTP or GDP standards with known isotope ratios, we assessed the accuracy of LC/oxidization/IRMS by comparing the isotope ratios determined for GDP and GTP of the same origin. A sample was prepared that consisted of $0.19 \mu\text{g}/\mu\text{l}$ GDP and $0.25 \mu\text{g}/\mu\text{l}$ GTP. The GTP in the sample was a mixture of ^{13}C -nucleotide and ^{12}C -nucleotide formulated at the natural carbon abundance ratio. GDP in the sample was derived from the same GTP through complete hydrolysis catalyzed by Ras and GAP. Therefore, the isotope ratios of GDP and GTP were expected to be the same and the ratio of their isotope ratios should be close to 1.0 within experimental precision. Indeed, in three measurements of $12.5 \mu\text{l}$ injections, the isotope ratios of GDP and GTP were determined, respectively, in units of atom percent (at%), as (1.0773, 1.0778), (1.0767, 1.0772), and (1.0773, 1.0770). The ratios of the isotope ratio of GDP to that of GTP were hence 0.9995, 0.9995, and 1.0003. The average deviation from 1.0 over the three measurements was -0.0002, demonstrative of the accuracy in isotope ratio measurement. The precision in isotope ratio measurement was represented by the standard deviations in isotope ratios of GDP (1.0773, 1.0767, and 1.0773) and GTP (1.0778, 1.0772, and 1.0770), which were 0.0003 (0.03%) and 0.0004 (0.04%), respectively.

Dynamic range of LC/oxidization/IRMS

In principle KIE can be measured at any point in the time course of the reaction. However, as the reaction proceeds, GTP is converted to GDP. At the extremes of f , the quantity of either GTP or GDP in the sample may exceed, or fall below, the level required for accurate measurement of isotopic ratio and peak area. Consequently, reactions must be sampled at reaction extents (f) within the range of nucleotide quantity over which R_p , R_s , and f can be accurately and precisely measured. To define the suitable dynamic range of f , we measured isotope ratios and peak areas of GDP and GTP in the sample that consisted of $0.19 \mu\text{g}/\mu\text{l}$ GDP and $0.25 \mu\text{g}/\mu\text{l}$ GTP at sample amounts of 2.5, 5, 7.5, 12.5, and 25 μl . As shown in panel (A) and (B) in Figure 3, a slight dependence of isotope ratio on the quantity of nucleotide was observed. However, the change in isotope ratio as predicted by the respective linear fit for GDP or GTP is less than 0.001 at% in the range between $1 \mu\text{g}$ and $6 \mu\text{g}$ nucleotide. The observed isotope ratios for samples containing less than $0.5 \mu\text{g}$ GDP were higher. Therefore, measurements of isotope ratio made with $0.5 \mu\text{g}$ or less nucleotide were deemed to be inaccurate.

Fractional reaction extent is calculated from the ratio of the peak area of GDP to the total area of GDP and GTP. Figure 3 shows excellent linear behavior of peak area with respect to sample quantity in the range from $1 \mu\text{g}$ to $4 \mu\text{g}$ nucleotide. Consequently, f is expected to be determined

accurately within this dynamic range. The precision in the determination of the ratio of peaks areas, as judged by the standard deviation of three individual determinations, was approximately 1% for each sample amount. For quantity of GDP exceeding 5 μg , peak area was slightly smaller than the linear trend. This could not be attributed to saturation of the detector or insufficient oxidation in the Isolink interface. The peak height was well within the linear range of the detector and the characteristic broadening of sample peak due to insufficient oxidation was not observed. We hypothesize that CO_2 separation unit in the Isolink cannot extract CO_2 quantitatively for samples containing more than 5 μg nucleotide.

In a typical sample, GDP and GTP were derived from 5 μg GTP as starting substrate and fractional reaction extent ranged from 0.2 to 0.8. Thus, the quantity of GDP or GTP under analysis ranged from 1 μg to 4 μg , where the accuracy and precision in the determination of both isotope ratio and f were optimal.

Practical considerations in measurement of isotope ratio

Accurate determination of isotope ratio requires quantitative conversion of carbon in GDP or GTP to CO_2 . If, for example, ^{13}C - and ^{12}C -nucleotides are oxidized at different rates, incomplete conversion potentially results in isotopic fractionation, such that the derived CO_2 gas has a different isotope ratio from that of the nucleotide mixture. To investigate whether the oxidizing condition in the Isolink was sufficient to quantitatively convert carbon to CO_2 , we carried out measurements at different concentrations of oxidizing reagents. Typically, analyte was oxidized by mixing of 1 M sodium peroxodisulfate and 1.5 M H_3PO_4 (with a catalytic amount of Ag^+) each at 50 $\mu\text{l}/\text{min}$ with HPLC effluent at 400 $\mu\text{l}/\text{min}$ and heating at 99.9°C. The isotope ratios of 5 μg natural GTP were observed to be 1.1397, 1.1397, 1.1402, and 1.1398 at% when flow rates of both solutions were set to 25, 50, 75, and 100 $\mu\text{l}/\text{min}$, respectively. Thus, the observed isotope ratio of GTP as determined by LC/oxidation/IRMS remained the same under increasingly more oxidizing conditions. We conclude that the oxidation condition used in our study was sufficient.

Isotopic fractionation of nucleotides during chromatographic separation can also affect the accuracy of isotope ratio determinations. That such fractionation occurs is evident from the variation in 45/44 ratio across the chromatographic peaks of GDP and GTP shown in panel (A) of Figure 2. Apparently ^{13}C -labeled nucleotide was eluted from the HPLC column one or two seconds before ^{13}C -depleted nucleotide. It is important to choose peak-picking parameters, namely the starting slope and the ending slope, so that a peak on mass 44, 45, or 46 curve is integrated in its totality. Starting and ending slopes define the starting and ending points and hence baseline, width, and area of a peak. To investigate whether the observed isotope ratio is sensitive to peak-picking parameters, we processed the particular chromatographic run in Figure 2 with different starting and ending slopes and tabulate the results in Table 1. The starting and ending slopes typically used in our studies, 0.2 and 0.4 mV/S respectively, constituted a robust set of parameters. Isotope ratio values did not change appreciably even when starting and ending slopes were varied by eight fold in either direction. This was true even for GTP, the peak length and peak area of which changed as much as a few percent with varying ending slope because of the long tail. This can be rationalized by the observation that isotopic fractionation occurs mainly in the bulk of the peak, not in the tail.

Another concern arises when the isotope ratio of a nitrogen-containing compound, such as a nucleotide, is measured. Nitrogen oxides exist in forms of N_2O , NO, N_2O_3 , NO_2 , and N_2O_5 . Of these gasses, N_2O and NO_2 can contribute to mass 44 ($^{14}\text{N}^{14}\text{N}^{16}\text{O}$), 45 ($^{14}\text{N}^{15}\text{N}^{16}\text{O}$), and mass 46 ($^{14}\text{N}^{16}\text{O}^{16}\text{O}$ and $^{14}\text{N}^{16}\text{O}^{18}\text{O}$). We are not aware of any study that investigates the chemical forms of nitrogen under the oxidizing condition in the Isolink. That the observed isotope ratio of natural GTP (1.14 at%) is close to natural abundance (1.11 at%) indicates that error introduced by nitrogen oxides, if present, is small. It is important to note that that it is the

ratio of isotope ratios (R_p/R_s , R_s/R_0 , and R_p/R_{100}), rather than their absolute values, that is used to calculate KIE. Small errors introduced by nitrogen oxides to isotope ratios are expected to cancel out when ratios are taken.

KIE for $[\beta^{18}\text{O}_3, ^{13}\text{C}]\text{GTP}$ in hydrolysis of GTP catalyzed by Ras and NF1³³³

For an irreversible enzymatic reaction such as Ras-catalyzed GTP hydrolysis [1], the observed KIE is a function of the intrinsic KIE in the chemical step and the forward commitment of the reaction [11]. Forward commitment is the partition function for Ras•GTP•NF1 poised for the chemical step and is defined as the ratio of the rate of the chemical step to the net rate of GTP release from this intermediate into solution. Mathematically, forward commitment behaves like a scaling factor that reduces the observed KIE relative to the intrinsic KIE. In order to infer the transition state structure, it is desirable to reduce the commitment factors as much as possible so that the observed KIE approaches the intrinsic KIE. For this purpose, we included a guanine nucleotide exchange factor for Ras, CDC25, to accelerate GTP release from Ras [26].

To demonstrate the accuracy and precision of the method, we tabulate in Table 2 the individual determinations of KIE for $[\beta^{18}\text{O}_3, ^{13}\text{C}]\text{GTP}$ in the hydrolysis of GTP catalyzed by Ras and NF1³³³. Three reactions were carried out. Each reaction mixture was divided into three aliquots and subjected to three chromatographic runs. For each run, KIE_p , KIE_s , and $KIE_{p/s}$ were calculated according to changes in the isotope ratio of the product (R_p/R_{100} ; equation 1), the substrate (R_s/R_0 ; equation 3) and the ratio of isotope ratios of product and substrate (R_p/R_s ; equation 2), respectively. Therefore nine values were obtained for each experiment. For each experiment, values of KIE_p , KIE_s , and $KIE_{p/s}$ were averaged separately, yielding three averaged values.

Precision of the method can be assessed by the standard deviations of the three KIEs calculated using the same equation within an experiment. The variations within the triplets originated from random errors in isotope ratio measurement, which was around 0.04% as reported in the previous section. The standard deviations were usually less than 0.1%, but in some instances were greater than 0.1% because errors in KIE depend on the method of computation as well and can be larger than errors in isotope ratio.

The accuracy of the method can be ascertained by comparing KIE_p , KIE_s , and $KIE_{p/s}$ averaged within one experiment or over all three experiments. Good agreement was observed between the average KIE_p , KIE_s , and $KIE_{p/s}$ for all three experiments –the standard deviations were 0.0003, 0.0006, and 0.0009 (not listed in Table 2), respectively. Excellent agreement was also found between KIE_p , KIE_s , or $KIE_{p/s}$ averaged over nine values in the three experiments, which were 1.0125, 1.0122, and 1.0121 with standard deviations of 0.0009, 0.0017, and 0.0006, respectively. The observed agreement demonstrates that all three ways of computing yield accurate KIE. However, it is important to note that the three ways of computing KIE are not equivalent. $KIE_{p/s}$ values had the smallest variations both within the experiment and between the experiments. The reason for this is two-fold. First, R_p/R_s is by definition the sum of R_s/R_0 and R_p/R_{100} , so the “signal” in R_p/R_s is larger than that in R_s/R_0 or R_p/R_{100} . Second, R_p and R_s are obtained in the same chromatographic run and some of the errors tend to cancel out in their ratio, so the “error” in R_p/R_s is lower. Therefore, we prefer to base our calculation of KIE values on R_p/R_s .

The variations observed among the three experiments, as judged by the averaged $KIE_{p/s}$ values of 1.0125, 1.0120, and 1.0118, can be accounted for by the errors in measurement. Since the purity of ^{13}C -depleted and ^{13}C -labeled nucleotides in the substrate mixture is critical to KIE measurement, the three experiments for $[\beta^{18}\text{O}_3, ^{13}\text{C}]\text{GTP}$ were carried out using substrate

mixtures that were independently mixed and purified. We conclude that that KIE measurement is reproducible for different preparations of substrate mixture.

It is also noted that the KIE for [^{13}C , $\beta^{18}\text{O}_3$]GTP includes isotope effects in both ^{13}C -labeling and ^{18}O -labeling. The contribution of ^{13}C to the overall isotope effect was determined to be 1.0048 (Table 2) using a substrate mixture consisting of ^{13}C -depleted nucleotide and ^{13}C -, but not ^{18}O -, labeled nucleotide. The isotope effect in $\beta^{18}\text{O}_3$ -labeling was found to be 1.0073, given by the ratio of $KIE_{p/s}$ for [$\beta^{18}\text{O}_3$, ^{13}C]GTP (1.0121, average of 1.0125, 1.0120, and 1.0118) and KIE for [^{13}C]GTP (1.0048).

Comparison to previous methods for the measurement of ^{18}O KIE in phosphoryl transfer reactions

Cleland and colleagues have developed double-labeling, internal competition methods to measure ^{18}O KIE in phosphoryl transfer reactions, including the hydrolysis reactions of phosphate monoesters, diesters, and trimesters [28] and phosphoryl transfer from ATP to glucose catalyzed by yeast hexokinase [17]. In these studies, the remote nitrogen atom in the *p*-nitrophenyl leaving group of phosphoesters or in the adenine group of ATP, was used as the “reporter” atom, which is isolated and converted to N_2 for IRMS analysis. This procedure is conducted offline and necessitates the use of large quantities of reactant or substrate (100 μmol *p*-nitrophenyl or 80 μmol ATP) for each experiment. The use of LC/oxidation/IRMS contributes to the ease of operation and high sensitivity of the method described here. The quantities necessary for KIE determination, namely the isotope ratios and peak areas of GDP and GTP, are measured in a single chromatographic run. The method is also highly sensitive in that each KIE determination requires only 5 μg (10 nmol) nucleotide. This high sensitivity should also allow the measurement of ^{18}O equilibrium isotope effects in the binding of substrate to enzyme and ^{18}O KIE under single turnover conditions.

Comparison of the CRI interface and the Isolink interface

Although the LC-coupled IRMS is ideally suited to the measurement of isotope ratio of soluble, nonvolatile compounds, which include most biologically active molecules, its application has lagged behind the analogous gas-phase technique, GC/IRMS. This can be attributed to the experimental challenges involved in coupling LC to IRMS. An interface is needed to convert the carbon atoms of the analyte quantitatively to CO_2 in continuous mode. The carbon dioxide must also be extensively dried to maintain the high vacuum in the spectrometer. Before the advent of the Isolink interface, the only working interface for LC-coupled IRMS was the chemical reaction interface (CRI) designed by Abramson and colleagues [29-31]. In a CRI, solvent is first removed by a nebulizer, a Universal Interface, and a momentum separator before analyte is converted into small molecules such as CO_2 , CO, N_2 , etc, in a microwave-powered helium plasma in the presence of O_2 . In earlier KIE studies of Ras-catalyzed GTP hydrolysis we employed a CRI/IRMS [32].

We believe that the CRI interface should remain a viable interface for LC-coupled IRMS. The two types of LC-IRMS interface complement each other with respect to their compatibility with different chromatographic systems. The CRI interface works ideally with reverse phase chromatography and common organic solvents such as acetonitrile and methanol. By contrast, the Isolink interface does not tolerate carbon in the solvent and is compatible with ion exchange or mixed mode chromatography where inorganic salts or acids may be used as eluting reagent [18,33]. The CRI interface is advantageous over the Isolink with respect to its versatility. The use of different reactant gasses in the CRI allows measurement of isotope ratio or detection of hydrogen, nitrogen, oxygen, sulfur, phosphorus, chlorine, bromine, and selenium [34]. Therefore, the CRI interface remains a very useful tool for KIE studies on reactions involving these elements either at site of chemical interest or as “reporter” site.

Conclusion

A highly sensitive and precise method has been developed to measure the ^{18}O KIE in the hydrolysis of GTP. The methodology described here is generally applicable to reactions involving other nucleotide triphosphates and will allow mechanistic studies on a large number of enzymes, including nucleotide triphosphatases, nucleotide cyclases, protein kinases, motor proteins, chaperones, and other proteins that couple nucleotide hydrolysis to work.

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Figure 1.

Schematic diagram of the ThermoFinnigan Isolink interface. HPLC effluent is mixed with oxidation reagents through a T joint. The carbon source is oxidized quantitatively into CO₂ in a heated oxidation reactor. In the CO₂ separation unit, CO₂ escapes from the aqueous phase through thin membranes into a countercurrent flow of helium. The helium stream carrying CO₂ is subsequently dried by two gas dryers (Nafion™) in series and admitted into IRMS.

Figure 2.

Panel (A) and (B) show the 45/44 ratio and channel 44 signal, respectively, for a typical chromatographic separation on the LC/oxidation/IRMS. Three pulses of CO₂ with known isotope ratios were introduced into the spectrometer at the beginning of the program to calibrate the measured isotope ratio. The CO₂ for HEPES buffer was not admitted into IRMS, resulting in a lower background between 200 to 500 second. The baselines for the peaks identified by the starting and ending slopes typically used in our studies, 0.2 and 0.4 mV/S respectively, are shown in the figure.

Figure 3.

The isotope ratios and peak areas of GDP and GTP were measured for a sample containing 0.19 $\mu\text{g}/\mu\text{l}$ GDP and 0.25 $\mu\text{g}/\mu\text{l}$ GTP at sample amounts of 2.5, 5, 7.5, 12.5, and 25 μl . Panel (A) and (B) show the isotope ratio (+) and peak area () versus sample amount for GDP and GTP, respectively.

Table 1

Statistics for integration of GDP and GTP peaks^a.

Slope (mV/S)		GDP			GTP		
Starting	Ending	Width (S)	Area (VS)	I.R. (at%)	Width (S)	Area (VS)	I.R. (at%)
0.2	0.05	98.3	58.79	1.0773	152.2	78.57	1.0772
0.2	0.4	97.8	58.78	1.0773	131.1	77.58	1.0770
0.2	3.2	78.9	58.07	1.0772	100.7	75.33	1.0769
1.6	0.4	90.5	58.62	10.771	127.1	77.57	1.0770
0.025	0.4	98.8	58.79	1.0773	131.1	77.58	1.0770

^aStatistics was obtained for the chromatographic separation of 2.4 µg GDP and 3.1 µg GTP shown in Figure 2. I.R. stands for isotope ratio.

Table 2
KIE determinations in GTP hydrolysis catalyzed by Ras and NF1³³³.

labels	<i>f</i>	<i>R_{p,obs}</i> (at%)	<i>R_{s,obs}</i> (at%)	<i>KIE_p^a</i>	<i>KIE_s^b</i>	<i>KIE_{p/s}^c</i>
¹³ C	0.000 ^d		1.0773 ^f			
	0.515	1.0743	1.0811	1.0042	1.0053	1.0049
	0.567	1.0744	1.0817	1.0043	1.0053	1.0050
	0.660	1.0749	1.0821	1.0040	1.0045	1.0045
	1.000 ^e	1.0771 ^f				
	mean			1.0042	1.0050	1.0048
	S.D.			0.0002	0.0005	0.0003
[¹³ C, β ¹⁸ O ₃]#1	0.000 ^d		1.1510 ^f			
	0.331	1.1421	1.1572	1.0116	1.0143	1.0118
	0.409	1.1408	1.1583	1.0140	1.0119	1.0130
	0.619	1.1434	1.1642	1.0137	1.0128	1.0127
	1.000 ^e	1.1520 ^f				
	mean			1.0131	1.0131	1.0125
	S.D.			0.0013	0.0012	0.0006
[¹³ C, β ¹⁸ O ₃]#2	0.000 ^d		1.1155 ^f			
	0.312	1.1000	1.1352	1.0122	1.0148	1.0123
	0.382	1.1024	1.1395	1.0130	1.0125	1.0122
	0.705	1.1036	1.1438	1.0121	1.0119	1.0115
	1.000 ^e	1.1145 ^f				
	mean			1.0124	1.0131	1.0120
	S.D.			0.0005	0.0015	0.0004
[¹³ C, β ¹⁸ O ₃]#3	0.000 ^d		1.0779 ^f			
	0.324	1.0682	1.0816	1.0113	1.0096	1.0113
	0.392	1.0682	1.0829	1.0119	1.0103	1.0118
	0.596	1.0698	1.0881	1.0123	1.0115	1.0123
	1.000 ^e	1.0772 ^f				
	mean			1.0118	1.0105	1.0118
	S.D.			0.0005	0.0010	0.0005
<hr/>						
	Mean (all 3)			1.0125	1.0122	1.0121
	S.D.			0.0009	0.0017	0.0006

^a KIEs calculated using Equation (3).

^b KIEs calculated using Equation (1).

^c KIEs calculated using Equation (2).

^d Substrate at 0% reaction.

^e Product of 100% reaction.

^f Average of three independent measurements.