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Measurement of Kinetic Isotope Effects in an Enzyme-Catalyzed Reaction by Continuous-Flow Mass Spectrometry

Kenneth M. Roberts[*], **Paul F. Fitzpatrick**[†,1]

[*]University of South Carolina Aiken, Aiken, SC, United States

[†]University of Texas Health Science Center, San Antonio, TX, United States

Abstract

Kinetic isotope effects (KIEs) provide powerful probes of the mechanisms of enzyme-catalyzed reactions. In this chapter, we describe the use of continuous-flow mass spectrometry to determine the deuterium KIE for the enzyme N-acetylpolyamine oxidase based on the ratio of labeled and unlabeled products in mass spectra of whole reaction mixtures.

1. INTRODUCTION

To fully determine the mechanism of an enzyme-catalyzed reaction, one must determine the structures of all intermediates and transition states. As discussed elsewhere in this volume, kinetic isotope effects (KIEs) provide arguably the most powerful method for analysis of mechanisms and determination of transition state structures. The methods for measuring KIEs are either competitive or noncompetitive, with all but deuterium isotopes generally being measured by competitive methods due to their higher precision. When the isotopes being used are not radioactive, the method of choice has been isotope ratio mass spectrometry (IRMS). While capable of impressive precision, IRMS has significant limitations. It is limited in the isotopes it can detect, with ^{15}N and ^{13}C being the most relevant for enzymological studies; this can be overcome by the use of a remote label, for example by using a remote ^{15}N substituent to measure an ^{18}O effect (Cleland, 1990). IRMS can also require a significant amount of material, although improved instrumentation has decreased that problem. Finally, the method requires that the product or remaining substrate be purified away from other compounds that contain the element of interest; this step can be the most demanding in that one must avoid any selective loss of one isotope versus another. For example, in isolating an amine product or substrate for measurement of an ^{15}N effect, the use of an ion-exchange column can result in partial separation of ^{14}N- and ^{15}N-labeled material due to the isotope effect on the amine pK_a. Several of these problems are avoided if the reaction mixture is injected without any work-up directly into a mass spectrometer using a continuous-flow system. With this approach, the mass spectrometer effectively carries out the separation of products and substrates. Since there are no steps between the reaction and the analysis, adventitious fractionation is avoided. The sensitivity of modern electrospray mass spectrometers means that the requirements are small in terms of material. We describe

[1]Corresponding author: fitzpatrickp@uthscsa.edu.

here a method for the measurement of a deuterium isotope effect on an enzyme-catalyzed reaction. The method should also be applicable to measuring effects with heavier atoms.

2. DEVELOPMENT OF A CONTINUOUS-FLOW MASS SPECTROMETRY SYSTEM

The continuous-flow method, in which two reagents are mixed and then passed through a tube containing a detector, was the first method developed for observing chemical and enzyme-catalyzed reactions on a millisecond time scale (Gutfreund, 1999; Hartridge & Roughton, 1923). While this methodology has generally been replaced by stopped-flow methods, the continuous flow approach is much more versatile in the range of detection methods one can use. The basic components of a continuous-flow mass spectroscopy (CFMS) system are pumps to drive solutions containing enzyme and substrate together, a mixer, and an electrospray mass spectrometer with a source connected to the mixer. The mass spectrometer thus serves as the detector, while the reaction time is varied by either changing the flow rate or changing the length of the tubing between the mixer and the source.

Our CFMS system is designed around a high-resolution mass spectrometer with a nanospray electrospray source incorporating a nanovolume chip mixer from Eksigent Technologies (Fitzpatrick, Chadegani, Zhang, Roberts, & Hinck, 2016; Gaweska, Roberts, & Fitzpatrick, 2012; Roberts, Tormos, & Fitzpatrick, 2014). Briefly, an HPLC pump pushes a sample containing enzyme and a sample containing substrate along separate lines to the chip mixer (Figure 1). The chip mixer contains a Peltier temperature controller to permit regulation of the reaction temperature. The mixture passes out of the chip mixer to a nanospray emitter affixed directly to the exit port of the chip mixer. In reactions where the substrate is unaffected by the application of voltage (such as for our assays with *N*-acetylpolyamine oxidase; PAO) (Roberts et al., 2014), the voltage tee for the electrospray is applied upstream of the chip mixer onto the line containing substrate. For applications in which the substrate is sensitive to the voltage field of the spray, the voltage tee can be applied between the chip mixer and the emitter. This does increase the delay volume significantly as the volume of the voltage tee is included in this arrangement.

Alternative systems have been described for CFMS of enzyme-catalyzed reactions. Anderson and co-workers (Paiva, Tilton, Crooks, Huang, & Anderson, 1997) utilized syringe pumps to drive reagents through a zero-volume mixer before injection into the electrospray mass spectrometer, although this system does not appear to have been used to measure isotope effects. Wilson and co-workers have described a continuous-flow mass spectrometer that uses a two-capillary system for mixing that varies the reaction time by altering the relative position of the capillaries and have recently applied it to measuring deuterium and ^{13}C isotope effects on enzyme-catalyzed reactions (Liuni, Olkhov-Mitsel, Orellana, & Wilson, 2013; Wilson & Konermann, 2003).

Our CFMS system is restricted to reaction times between 0.6 and 24 s. Different reaction times are achieved by varying the flow rate. A total flow rate of 0.25 μL/min yields a reaction time of 24 s; we have found that total flow rates less than 0.75 μL/min result in

significant problems with electrospray quality when using emitters with 10 μm tips. This may be overcome using emitters with 5 μm tips, although the smaller tip diameter can result in greater tip heterogeneity and more potential for obstruction. An alternative for increased reaction times would be to insert fused-silica tubing between the mixer and the emitter. A total flow rate of 10 μL/min (5 μL/min for each sample line) is the maximum achievable with the NanoLC pump, giving a minimum reaction time of 0.6 s. While it would be possible to replace the pump with one capable of higher flow rates, we have found that total flow rates of 5 μL/min or greater result in increasingly heterogeneous electrosprays. Emitters with larger tips (20 or 50 μm) could be used instead at these higher flow rates; this will result in an increased delay volume.

2.1 Experimental Considerations

A significant limitation in analyzing enzymatic reactions by CFMS is that the buffers typically used for enzymes can be incompatible with electrospray mass spectrometry. Ammonium acetate has adequate buffer capacity at pH 8.5 and is a volatile salt. At pH 7–8, a buffer composed of 1,2-diammoniumethylene diacetate (EDDA) and ammonium chloride can be used (Fitzpatrick et al., 2016; Roberts et al., 2014)). EDDA is a volatile salt with a pK_a of 7.6. The addition of 5% methanol to the reaction substantially increases the quality of the spray and can be required to obtain acceptable spectra; presumably other organic solvents miscible with water could be used, but methanol has the advantage of low-cost. Typically, the enzyme is prepared in buffer without methanol, and the substrate is prepared in buffer plus 10% methanol. Mixing these solutions at equal volumes gives 5% methanol in the final reaction mixture. On the time-scale of the reactions described here, the exposure of an enzyme to this concentration of methanol generally has little effect on structure or activity.

2.2 Calibration of the System

In continuous-flow assays, the reaction time is dependent on how long the reaction mixture takes to flow from the mixer to the detector (Gutfreund, 1999; Hartridge & Roughton, 1923). The reaction time (t), then, is dictated by the flow-rate of the mixture (Q) and the volume of the delay line (V) (Equation 1). The electrospray source reduces the droplet size of the solvent mixture until the individual ions are fully desolvated (Cole, 2000); the enzymatic reaction can take place within the droplets until the reaction components are separated immediately prior to detection. The ‘delay line’ of the CFMS system, therefore, includes everything from the mixer (reported to us as 40 nL), on through the emitter (10 nL), and well into the electrospray, until all of the components are isolated and enter the mass spectrometer as individual ions. Consequently, it is necessary to determine an apparent delay volume (V_{app}) for a given mixer and source arrangement.

$$t = \frac{V}{Q} \quad \text{Equation 1.}$$

The available reactions times were taken into consideration in selecting the enzyme-catalyzed reaction for initial characterization. Specifically, a reaction that would be 80–90%

complete in 10–20 s was desired. We had previously analyzed the reaction of PAO with several substrates (Henderson Pozzi, Gawandi, & Fitzpatrick, 2009). The reaction with one, *N,N'*-dibenzyl-1,4-diaminobutane (DBDB, Figure 2), had appropriate kinetic parameters (k_{cat} = 0.8 s^{-1}, K_m = 15 μM) at pH 8.6 for the time scale we were interested and consequently was selected.

Quantitative mass spectrometry requires the use of internal standards to account for the variation in the analyte signal with conditions. Critically, the ion intensity for a specific analyte varies with the total flow rate of the electrospray (Hopfgartner, Wachs, Bean, & Henion, 1993; Page, Kelly, Tang, & Smith, 2007); this flow-dependent variation is unique for each analyte. For the PAO reaction, it was thus necessary to find an analogue of DBDB that shows a similar flow-dependent signal profile. To do so, potential analogues and DBDB were mixed with buffer in the absence of enzyme and injected into the mass spectrometer. The relative intensities of the ions for DBDB and the analogue were then compared at multiple sample flow-rates. We found that *N*-methylphenethylamine (MPEA) exhibited a change in intensity with flow rate comparable to that observed for DBDB.

To determine the V_{app} of our CFMS system, the time course for the oxidation of DBDB by PAO was first determined directly under the buffer and temperature conditions to be used for CFMS. The concentrations of DBDB (40 μM) and PAO (20 μM) were chosen such that ~90% of the DBDB was consumed over the first 15 s of the reaction and the signal-to-noise for DBDB was acceptable. We used a BioLogic QFM-400 quenched-flow apparatus to mix 80 μM DBDB plus 80 μM MPEA, in 10% methanol, 50 mM ammonium acetate, pH 8.5, with 40 μM PAO in 50 mM ammonium acetate, pH 8.5, at 30 °C, quenching the reaction with 2 M HCl after 50 ms to 10 s. The amount of DBDB remaining at each time was then determined by HPLC. Since the initial concentration of DBDB was close to the K_m value, the change in the concentration with time was reasonably well described by equation 2, yielding the value for the first order rate constant (k_{obs}) for the reaction under these conditions. The reaction was then repeated in the CFMS system under the same conditions, measuring the relative ion intensity of DBDB across a range of flow rates, using MPEA as an internal standard. The CFMS data were fit to equation 3 using the value of k_{obs} determined from the chemical-quench analyses (Figure 3) to obtain the V_{app} value. For two different chip mixers, we obtained values for V_{app} of 102 ± 7 and 210 ± 20 nL, respectively, when the CFMS system was set up as shown in Figure 1.

$$[\mathrm{DBDB}]_t = [\mathrm{DBDB}]_0 e^{-k_{obs} t} \quad \text{Equation 2.}$$

$$[\mathrm{DBDB}]_t = [\mathrm{DBDB}]_0 e^{-k_{obs} \frac{V_{app}}{Q}} \quad \text{Equation 3.}$$

2.2.1 Equipment

- Thermo Scientific LTQ OrbiTrap Discovery

- New Objective PicoView nanospray source with Eksigent Technologies Nanovolume Chip Mixer
- Fused-silica tubing (360 μm OD, 150 μm ID)
- New Objective Picotip distal coated SilicaTip emitter (20 μm ID, 10 μm tip)
- Bio-Logic Science Instruments QFM-400 quenched-flow system
- Phenomenex Gemini-NX C18 HPLC column (3.0 μm, 1.0×150 mm) and HPLC with fluorescence detector
- Synergy Software KaleidaGraph graphing and data analysis software

2.2.2 Buffers and Reagents

- *N*,*N*′-dibenzyl-1,4-diaminobutane (DBDB)
- *N*-methylphenethylamine (MPEA)
- *N*-acetylpolyamine oxidase (PAO)
- Buffer A: 50 mM ammonium acetate, pH 8.5
- Buffer B: 10% methanol in 50 mM ammonium acetate, pH 8.5
- Sample Solvent: 0.1% trifluoroacetic acid in water
- Elution Solvent: 0.1% trifluoroacetic acid, 17.5% acetonitrile in water

2.2.3 Procedure – CFMS Assays

1. Set-up the CFMS system as shown in Figure 1:
   a. Connect the LC pump separately to two six-port injection valves and the valves to the chip mixer using fused-silica tubing.
   b. A few centimeters before the chip mixer, insert the nanospray voltage tee onto the line from Loop B.
   c. Mount the sample loops onto the injection valve such that water from the pump may pass either directly through the valve to the chip mixer or through the sample loops to the chip mixer.
   d. Cut nanospray emitter to appropriate length and connect directly to the chip mixer.
2. Start the LC pump at the chosen flow rate to establish steady, even flow throughout the valves, sample lines and the chip mixer, generating a stable electrospray.
3. Load sample loops A and B with Buffer A (with or without enzyme) and 80 μM DBDB, 80 μM MPEA in Buffer B, respectively. Allow 2–5 min for the sample flows and pressure to reach an even, steady flow.
4. Once the sample flow has equilibrated, record 100 or more scans of the mass spectrum of the resulting reaction mixture in the range of m/z 130–300. Average

the ratio of the ion intensities for DBDB ($[M+H]^+$ 269.2012) and MPEA ($[M+H]^+$ 136.1121) across all scans.

2.2.4 Notes

1. The voltage tee *should not* be placed in line with the protein sample upstream of the chip mixer. We found that this arrangement leads to immediate precipitation of the protein within the tee, resulting in complete blockage of the tee within minutes.
2. We use nanospray emitters with a distal conductive coating because the coating assisted with the formation of a successful electrospray at the low concentration of methanol used in these assays.
3. Successful electrospray conditions for reaction analyses included a spray voltage of 4–6 kV when the voltage tee was applied upstream of the chip mixer. Lower voltages resulted in no spray. Higher voltages were complicated by erratic spray and background electrochemistry involving the substrates.
4. When applied downstream of the chip mixer, spray voltages as low as 2 kV could be applied to the source for successful electrospray. However, this arrangement results in a significant increase in the delay volume of the system.
5. At higher flow rates (greater than 0.5 μL/min per line), the pressure in the two sample lines is high enough that varying from a 1:1 ratio of flow rates between the sample lines is possible. This can allow varying the concentrations of enzyme and substrate in the reaction.
6. At lower flow rates (less than 0.5 μL/min per line), the flow rates of the lines *must* be equal, otherwise the pressure of the slower line can become overpowered by the faster line, preventing adequate mixing within the mixer. This results in a lower concentration of the slower flow rate sample in the mixture than expected.
7. Allowing the system to reach a steady, even pressure/flow is imperative. It takes 2 to 5 minutes for the sample lines, mixer, and emitter to equilibrate such that the flow rate is equal throughout the system. Since flow rate dictates the reaction time in this fixed-volume system, waiting until the flows stabilize ensures the mass spectra correlates to the correct reaction time.
8. To correct for the change in signal intensities at varying flow rates, samples include an internal standard (MPEA). The mass spectra of reaction samples are recorded for 100–200 scans and the ratio of the signal intensities of the analyte and the internal standard are averaged across all scans. Where possible, these ratios are compared to a standard curve.

2.2.5 Procedure – V_{app} Calibration

1. Using a quenched-flow apparatus, mix 50 μL 40 μM PAO in Buffer A with 50 μL 80 μM DBDB, 80 μM MPEA in Buffer B at 30 °C and quench with 50 μL 2 M HCl. Vary reaction times from 50 ms to 10 s.

2. Centrifuge the quenched samples at 12,000×*g* for 60 s to remove denatured protein. Add 100 μL of reaction supernatant to 900 μL Sample Solvent.

3. Quantify DBDB by injecting 20 μL of each sample onto a Phenomenex Gemini-NX C18 HPLC column with Elution Solvent as the mobile phase at a flow rate of 0.25 mL/min. The fluorescence detector is set at 278 nm with an excitation wavelength of 255 nm. The concentration of DBDB is determined from the fluorescence intensity of DBDB relative to that of the MPEA internal standard.

4. Determine the observed rate constant, k_{obs}, from a fit of the concentration of DBDB vs time with equation 2.

5. Repeat the reaction on the CFMS system by mixing 40 μM PAO in Buffer A with 80 μM DBDB, 80 μM MPEA in Buffer B at equal flow rates for a range of total flow rates (Q: 0.25 to 10 μL/min). For each flow rate (Q), average the mass spectrum (m/z 130 – 300) across 100 scans and calculate the concentration of DBDB using the MPEA internal standard.

6. Determine the apparent delay volume (V_{app}) from a fit of the concentration of DBDB vs Q with equation 3.

2.2.6 Notes

1. An important aspect of this procedure is that the reaction is performed under the same conditions in both methods. This eliminates any condition-dependent variances and allows direct comparison of the time-dependent concentration of the analyte (DBDB) by both methods.

2. Only the quality of the fit is important for the success in determining V_{app}. The nature of that change is not important. Although a ratio of 2:1 for DBDB:PAO is not truly first order, the data were well-fit with a first order rate equation and thus the fit could be used as an appropriate representation of both reactions.

3. MEASUREMENT OF KINETIC ISOTOPE EFFECTS

The ability to follow the PAO reaction by CFMS provided an opportunity to determine isotope effects on the reaction by a competitive method without the need for synthesis of multiply-labelled substrates or isolation of the product or remaining substrate at different reaction times. The product of the oxidation of DBDB by PAO is DBDB monoimine (DBDBi, Figure 2). To determine the $^{D}(k_{cat}/K_{m})$ value, we used a mixture of DBDB and DBDB-d_{14} as substrate and followed the formation of the products from the deuterated (m/z = 267.1856) and nondeuterated (m/z = 280.2672) substrates simultaneously using CFMS (Figure 4). The reactions contained DBDB-d_{14} and DBDB in a 5:1 ratio so that the intensities of the ions from the labeled and unlabeled substrates would be comparable. A separate analysis of the isotope effect using a non-competitive approach yielded a value of 6.7 ± 0.6; in the absence of prior knowledge of the isotope effect, one could vary the ratio of the labeled and unlabeled substrate to optimize the result. No internal standard was required, since the isotope effect was determined from the ratio of the labeled and unlabeled products relative to the substrate ratio at the beginning of the reaction, and isotopic substitution is not

expected to affect the ionization properties of the substrate and products. We tested this across a wide range of flow rates and found the relative ion intensities of the substrates to be independent of the flow rates. Figure 5 shows the product isotope effect as a function of time. Extrapolation of the time-dependent isotope effect to t_0 using equation 4 yielded a value of 7.7 ± 0.3 for $^{D}(k_{cat}/K_m)$.

$$\frac{\left(\frac{[\text{DBDBi}]}{[\text{DBDBi}-d_{13}]}\right)_t}{\left(\frac{[\text{DBDB}]}{[\text{DBDB}-d_{14}]}\right)_0} = \text{KIE}\, e^{-kt} \quad \text{Equation 4.}$$

3.1 Equipment

- CFMS system

3.2 Buffers and Reagents

- *N,N'*-di-(perdeuterobenzyl)-1,4-diaminobutane (DBDB-d_{14}) (Henderson Pozzi, Gawandi, & Fitzpatrick, 2009)

3.3 Procedure

1. Perform the CFMS reaction by mixing 80 μM PAO in Buffer A with 6.7 μM DBDB, 33 μM DBDB-d_{14} in Buffer B at 30 °C at equal flow rates such that total flow rates yield reaction times from 0.6 – 24 s.
2. For each reaction time, record the mass spectrum (m/z 130 – 300) for 100 scans. Calculate the observed product isotope effect for each time point as the average of the ratio of the ion intensities for DBDBi ($[M+H]^+$ 267.1861) and DBDBi-d_{13} ($[M+H]^+$ 280.2672) across all 100 scans.
3. Fit the time-dependence of the observed isotope effect to equation 4 to determine the isotope effect as the calculated value at time zero.

4. CONCLUSIONS

Kinetic isotope effects on an enzyme reaction can be measured using continuous-flow mass spectrometry. Important considerations for successful CFMS assays include a buffer system amenable to both an enzyme reaction and electrospray ionization mass spectrometry, quantification of analytes using an appropriate internal standard, accurate reaction times obtained from calibration of the flow rate and the apparent delay volume, and the starting conditions of the reaction.

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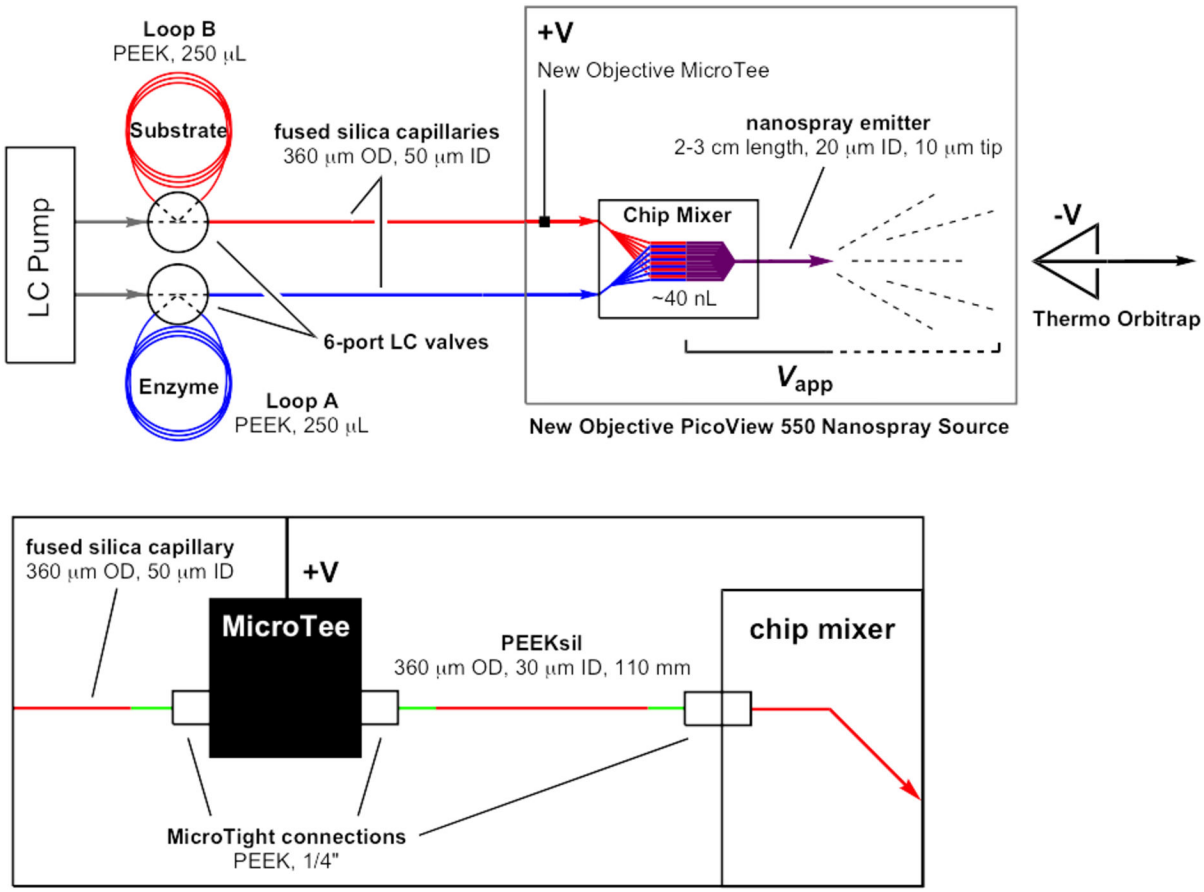

Figure 1.

Schematic of the continuous-flow mass spectrometry system used in our studies. The location of the voltage tee is indicated with +V. V_{app} represents the apparent delay volume.

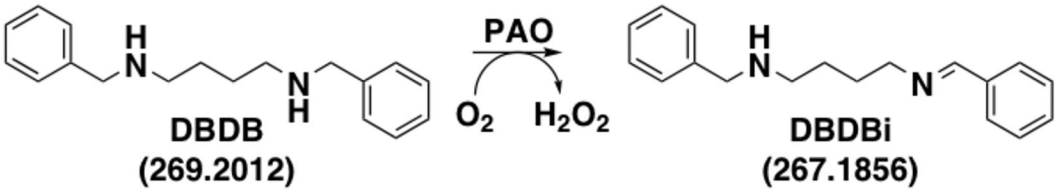

Figure 2.
Oxidation of DBDB by PAO.

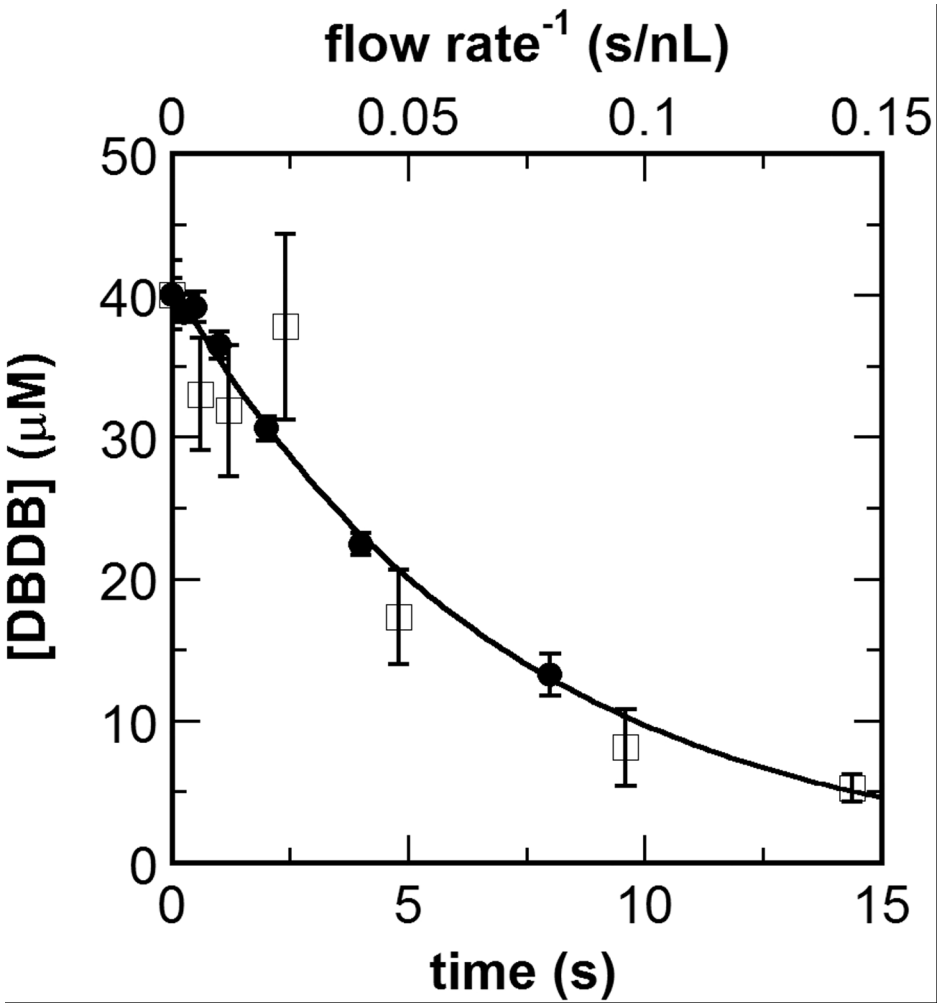

Figure 3.

Time-dependence of the oxidation of DBDB by PAO by chemical-quench (closed circles) and CFMS (open squares). Final reaction conditions were 40 μM DBDB, 20 μM PAO, 50 mM ammonium acetate, 5% methanol, pH 8.5, and 30 °C. The line indicates the best-fit curve for the chemical-quench data to equation 2 and the best-fit curve for the CFMS data to equation 3. The figure is from Roberts et al., 2014, http://pubs.acs.org/doi/full/10.1021/bi500267c.

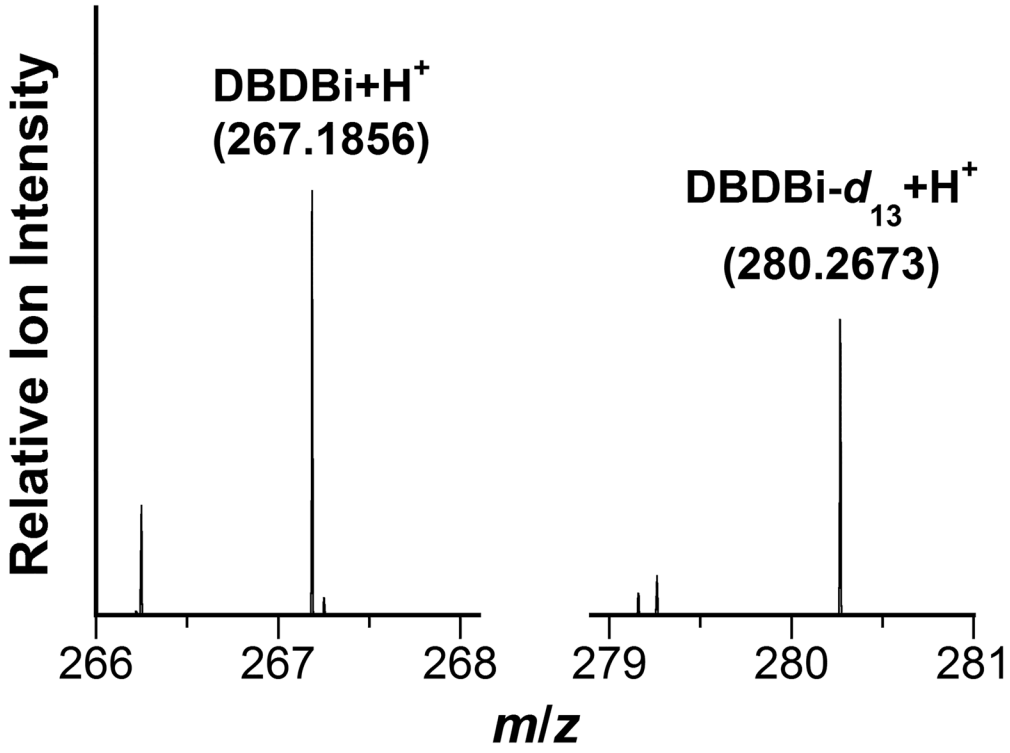

Figure 4.

Mass spectrum of the reaction of 40 μM PAO with 6.7 μM DBDB, 33 μM DBDB-d_{14} at 30 °C at a flow rate of 10 μL/min, for a reaction time of 1.3 s. The spectrum is an average of 100 scans.

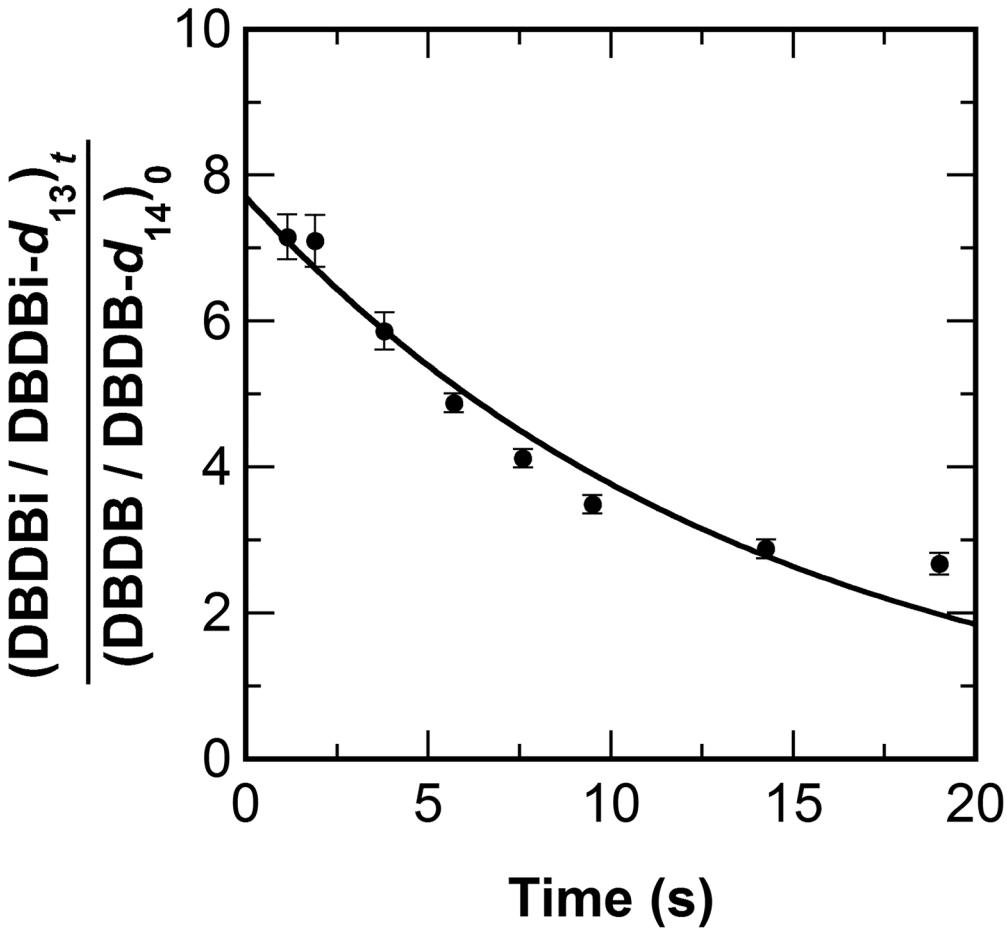

Figure 5.

Measurement of the deuterium isotope effect for oxidation of DBDB by PAO using continuous-flow mass spectrometry. The reaction contained 6.7 μM DBDB, 33 μM DBDB-d_{14}, and 40 μM PAO in 5% methanol and 50 mM ammonium acetate (pH 8.5) at 30 °C after mixing. The line is from a fit to equation 4. The figure is adapted from reference (Roberts et al., 2014), http://pubs.acs.org/doi/full/10.1021/bi500267c.