Communications to the Editor

Carbon Isotope Effects on k_{cat} for Formate Dehydrogenase Determined Using a Continuous-Flow Stirred-Tank Reactor

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Mechanistic investigations of enzyme-catalyzed reactions often include studies of heavy-atom kinetic isotope effects. Typically, competitive methods involving either isotope-ratio mass spectrometry or scintillation counting are used to measure the small effects on reaction rates arising from isotopic substitution at atoms of carbon, oxygen, phosphorous, or nitrogen within a substrate molecule.[1] In these experiments, isotopic ratios of reactants or products are measured at various extents of reaction as isotopic substrates compete for the enzyme. Owing to the competitive nature of the experiments, the resulting isotope effects are necessarily effects on rate constants (k_{cat}/K_M) corresponding to reactant states in which the isotopic substrates are free from the enzyme. Competitive measurements cannot provide isotope effects on k_{cat}, the rate constant for enzyme-catalyzed reactions when the catalyst is saturated with substrate. Instead, direct comparisons must be made for k_{cat} determined in separate experiments for isotopic substrates.

We have improved on the direct-measurement method for measuring k_{cat} isotope effects through the use of a continuous-flow stirred-tank reactor (CSTR).[2] We chose to test our method using the yeast formate dehydrogenase[3] reaction, because it was expected that hydride transfer/decarboxylation would be fully rate limiting for k_{cat}.[4,5] The formate dehydrogenase reaction is also well-suited as a test case for our method because it shows strong product inhibition by NADH. Consequently, high-precision k_{cat} measurements using conventional batch initial-velocity methods are particularly difficult in the case of formate dehydrogenase.

The reactor setup is shown in Figure 1, and a sample isotope-effect measurement is shown in Figure 2. At the start of the experiment, enzyme, NAD^+, and a saturating concentration of ^{12}C-formate were pumped into the reactor. Once the enzymatic reaction rate balanced the flow rate (material is flowing into and out of the reactor), a steady state was reached at point I on Figure 2. The enzymatic reaction rate for ^{12}C-formate is proportional to the steady-state absorbance (NADH) at 340 nm. The formate feed was next switched to a solution containing only ^{13}C-formate, and because there is an isotope effect on the reaction, a new steady state was reached (point II). The absorbance at this point is proportional to the ^{13}C-formate rate, but at a slightly different [NADH] (and [formate]) than the rate for ^{12}C-formate at steady-state I. The flow rate was then changed to allow the steady-state absorbance to match the value for the ^{12}C-formate steady state. The ratio of the flow rates at points I and III is the isotope effect on the reaction rate at identical substrate and product concentrations for the two isotopomers of formate.[6]

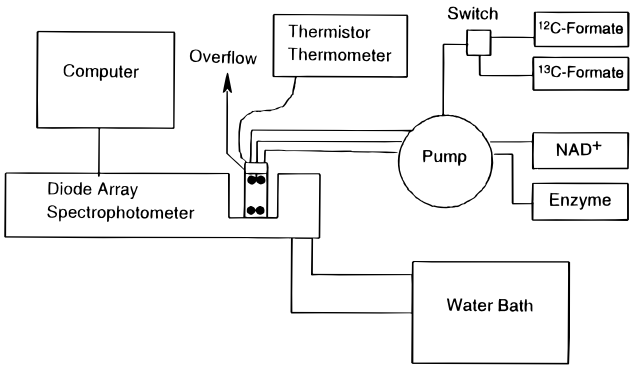

Figure 1. Continuous-flow stirred-tank reactor for enzyme-catalyzed reactions. The reactor was stirred from below using a magnetic stirrer and from above using a small direct-drive stirrer. The overflow was removed using a tube connected to an aspirator. The reactor volume was 3.59 mL under operating conditions. The pump (peristaltic) was a Rainin Rabbit-Plus; the UV−vis spectrophotometer was a Hewlett-Packard model 8452a.

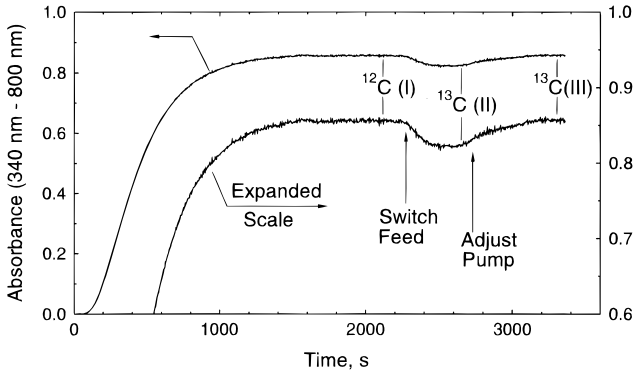

Figure 2. Sample run for the measurement of a carbon isotope effect. At the start of the experiment, the reactor was filled with water. As the enzyme, NAD^+ and ^{12}C-formate entered the reactor, NADH was produced (340 nm). After a steady state was reached (point I), the feed was switched to allow ^{13}C-formate to displace ^{12}C-formate in the reactor. Because there is an isotope effect on the reaction, a new steady state was observed (II). The pump flow rate was adjusted to match the ^{12}C and ^{13}C steady states (points I and III).

(1) Parkin, D. W. In *Isotope Effects from Enzyme Mechanism*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL, 1991; Chapter 11.
(2) Nauman, E. B. *Chemical Reactor Design*; Wiley: New York, 1987.
(3) Yeast formate dehydrogenase from *Xylaria digitata (Candida boidinii)* was used as obtained (lyophilized powder) from Boehringer Mannheim.
(4) Blanchard, J. S.; Cleland, W. W. *Biochemistry* **1980**, *19*, 3543−3550.
(5) Hermes, J. D.; Morrical, S. W.; O'Leary, M. H.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5479−5488.
(6) Typical amounts of material needed to operate the reactor over a 5 h period (enough time to measure three or four individual isotope effects) were 50 mL of each of the sodium formate solutions (120 mM), 100 mL of 6 mM NAD^+, and 100 mL of formate dehydrogenase (0.25 mg/mL). These values are the concentrations and volumes of the feed solutions for the reactor.
(7) The enzymatic rate can be obtained from rate = (flow rate)$A/(V\epsilon)$ where V is the reactor volume and ϵ is the extinction coefficient for NADH. Dividing the rate by the enzyme concentration when the substrate is at saturating levels gives k_{cat}. Enzyme concentration, reactor volume, and the extinction coefficient are identical for the ^{12}C and ^{13}C substrates.
(8) In a few instances, we have noticed bubbles trapped in the reactor. If bubbles cannot escape, the volume of the reactor (and therefore the residence time) will change making it difficult to reach a good steady state.

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Table 1. Carbon Isotope Effects on k_{cat} for the Yeast Formate Dehydrogenase Reaction[a] at 25 °C

run	substrate	absorbance[b] 340−800 nm	flow rate, μL/s	$^{13}k_{cat}$ (apparent)	correction	$^{13}k_{cat}$
XW024801	^{13}C	0.7939	10.376	1.0408	0.9998	1.0406
	^{12}C	0.7934	10.806			
XW019301	^{12}C	0.6390	17.023	1.0375	0.9999	1.0374
	^{13}C	0.6392	16.404			
HX049104	^{12}C	0.8569	14.943	1.0414	1.0001	1.0415
	^{13}C	0.8576	14.338			
HX049707a	^{12}C	0.7107	16.068	1.0405	0.9995	1.0400
	^{13}C	0.7117	15.422			
HX049707b	^{13}C	0.7117	15.422	1.0409	1.0004	1.0413
	^{12}C	0.7110	16.068			

[a] Reactions were carried out in 100 mM EPPS buffer at pH 7.80. Enzyme concentrations were ca. 0.08 mg/mL (in the reactor). The enzyme feed solution contained 0.1 mM each of EDTA and DTT. Steady-state reactor concentrations of NAD^+ varied from 1.8 to 2.1 mM for the runs shown in the table. For formate, the steady-state concentrations varied from 34 to 39 mM. [b] Steady-state absorbances (A_{ss}) were determined by least-squares fitting of absorbance (A) vs time (t) near the steady state to $A = A_{ss} + be^{-ct}$. Small contributions to the absorbance at 340 nm from NAD^+ will only influence isotope effects through the correction factor, and in all cases here the influence is negligible.

To account for the fact that the match between the absorbances at steady states I and III is never perfect, we describe (eq 1) the isotope effect on k_{cat} in terms of an apparent isotope

$$\frac{k_{cat,12}}{k_{cat,13}} = {}^{13}k_{cat} = \overbrace{\frac{(\text{flow rate})_{12}}{(\text{flow rate})_{13}}\frac{A_{12}}{A_{13}}}^{\text{apparent } {}^{13}k_{cat}}\underbrace{\frac{(mA_{13}+b)}{(mA_{12}+b)}}_{\text{correction}} \qquad (1)$$

effect and a correction. Flow rates were determined by measuring the mass of distilled water (known density) delivered by the pump at a particular setting over a period of 10−20 min. The product of the flow rate and the absorbance are directly proportional to the rate of the enzymatic reaction, and to k_{cat} when the enzyme is saturated with substrate.[7] The correction is based on linear interpolations of enzymatic rates vs absorbance using either ^{12}C or ^{13}C substrates. The interpolation parameters, m and b, were determined for each isotope-effect experiment using the steady states at points II and III in the figure. The correction, a ratio of reaction rates, accounts for the slight differences in steady-state absorbances realized for the ^{12}C and ^{13}C substrates. The procedure for making the correction assumes that over small changes in the extent of the enzymatic reaction, the changes in reaction rate are linear. To test whether systematic variations in the pump rate might influence the accuracy of the measurements, the sequence of isotopic formate feeds used was reversed in some experiments (the first and last entries in Table 1). The isotope effects are independent of the isotopic formate feed sequence, demonstrating that systematic variations in the pump rate are not a problem over the time course of individual isotope-effect measurements.

The average of five out of six $^{13}k_{cat}$ measurements (Table 1) is 1.0401 ± 0.0020 (95% confidence limits). One result was rejected because a constant final steady-state absorbance could not be reached before feed solutions were consumed.[8] Our $^{13}k_{cat}$ is not significantly different from $^{13}(k_{cat}/K_M) = 1.0423 \pm 0.0013$ (95% confidence limits) reported by Blanchard and Cleland.[4] The similarity between $^{13}k_{cat}$ and $^{13}(k_{cat}/K_M)$ provides additional support for the conclusion that the hydride-transfer/decarboxylation step is the sole rate-limiting step in the yeast formate dehydrogenase reaction.[4,5] The very similar kinetic isotope effects are also consistent with the absence of significant equilibrium substrate-binding isotope effects[9,10] for the reaction, in agreement with the small, near unity carboxylate-carbon isotope effects on binding inferred from kinetic isotope effects on pyruvate-kinase and pyruvate-carboxylase reactions.[11]

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(9) LaReau, R. D.; Wan, W.; Anderson, V. E. *Biochemistry* **1989**, *28*, 3619−3624.
(10) Gawlita, E.; Paneth, P.; Anderson, V. E. *Biochemistry* **1995**, *34*, 6050−6058.
(11) Gawlita, E.; Caldwell, W. S.; O'Leary, M. H.; Paneth, P.; Anderson, V. E. *Biochemistry* **1995**, *34*, 2577−2583.