A Critical Test of the "Tunneling and Coupled Motion" Concept in Enzymatic Alcohol Oxidation

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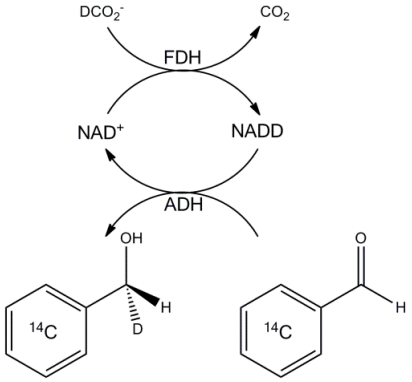
Supplementary Information

Experimental Details

Materials

All chemicals were from Sigma unless otherwise noted. [^{3}H]-$NaBH_4$ (350 mCi/mmol) was from American Radiolabeled Chemicals. Phenyl-[^{14}C]-benzaldehyde (55 mCi/mmol) was from ViTrax. [^{2}H]-$NaBH_4$ was from Cambridge Isotope Labs. 7-[^{3}H]-benzyl alcohol (racemic) was prepared by reduction of benzaldehyde by [^{3}H]-$NaBH_4$. 7-[^{3}H],[^{2}H]-benzyl alcohol (racemic) was prepared by reduction of benzoyl chloride by a mixture of [^{3}H]-$NaBH_4$ and [^{2}H]-$NaBH_4$. Phenyl-[^{14}C]-benzyl alcohol was prepared by reduction of phenyl-[^{14}C]-benzaldehyde by $NaBH_4$. 7*R*-[^{2}H]-phenyl-[^{14}C]-benzyl alcohol was prepared by a coupled synthesis depicted in scheme S1. The synthesis of this material has been of interest to various groups for over 10 years, and is relevant to broader application than the current paper, thus the details of the synthesis will be published elsewhere.[1] Briefly, the reduced form of 4*R*-[^{2}H]-nicotinamide adenine dinucleotide (NADD) was produced *in situ* by reduction with formate-d, catalyzed by formate dehydrogenase (FDH). The resultant NADD was used to reduce phenyl-[^{14}C]-benzaldehyde, catalyzed by horse liver ADH, yielding the desired 7*R*-[^{2}H]-phenyl-[^{14}C]-benzyl alcohol. The 500 µl reaction contained 100 mM phosphate (pH 7.5), 140 mM DCO_2Na, 10 mM phenyl-[^{14}C]-benzaldehyde (2 mCi/mmol), and 0.1 mM NAD^+. The reaction was initiated by the simultaneous addition of pre-mixed FDH from *Candida boidinii* and hlADH such that the final concentration of both enzymes was approximately 1 U/ml. The reaction was incubated at room temperature and reached complete conversion to products (i.e. quantitative conversion of benzaldehyde to benzyl alcohol) overnight.

Yeast alcohol dehydrogenase (yADH) isozyme 2 was expressed and purified according to a published procedure[2] using a strain of yeast deficient for other isozymes of ADH (a generous gift from Bryce V. Plapp, Dept. of Biochemistry, University of Iowa).



Scheme S1: Synthesis of 7*R*-[^{2}H]-phenyl-[^{14}C]-benzyl alcohol.

Kinetic Measurements

We measured 2˚ H/T KIEs with both H-transfer and D-transfer using competitive methods and conditions as described before.[3-5] Briefly, in such measurements both the light and heavy substrate react in the same container, competing for the enzyme's active site.[6,7] Competitive methods give very precise measurements, but generally require radiotracers for both substrates. Our methods used ^{3}H in the heavy substrates and the light substrates (which had either ^{1}H or ^{2}H) always had a remote ^{14}C label. To conduct these experiments, the heavy and light substrates must be co-purified and subsequently added to the reaction mixture. For the measurement of the 2˚ H/T KIE with H-transfer, 7-[^{3}H]-benzyl alcohol (racemic) was copurified with phenyl-[^{14}C]-benzyl alcohol. For 2˚ H/T KIE with D-transfer, 7-[^{3}H],[^{2}H]-benzyl alcohol (racemic) was copurified with 7*R*-[^{2}H]-phenyl-[^{14}C]-benzyl alcohol. The copurified alcohol was added to 1 ml reaction mixtures that contained 10 mM NAD^{+}, 300 mM semicarbazide, 80 mM glycine (pH 8.5), and 1 mM benzyl alcohol (300,000 dpm ^{14}C and 3,000,000 dpm ^{3}H). These conditions are based on those used in previous studies of yADH.[3-5] Reactions began after the addition of approximately 1 mg yADH and were incubated at 25˚ C. At a range of timepoints, we removed 100 µl aliquots and quenched them by the addition of 10 µl saturated $HgCl_2$. We stored the quenched aliquots at room temperature until ready for analysis by HPLC. The addition of $HgCl_2$ generated a precipitate, which we removed by centrifugation prior to HPLC analysis.

The semicarbazide in the reaction mixture traps the product benzaldehyde as benzaldehyde semicarbazone, which ensures that the reaction is irreversible. We separated the reactants and products at a range of time points by HPLC and analyzed the reactant and product fractions for ^{3}H and ^{14}C contents by LSC. We then calculated KIEs according to[6]

$$KIE = \frac{\log(1-f)}{\log\left(1-f*\frac{Rp}{R_\infty}\right)} \qquad [S1]$$

where R_p is the ratio of ^{3}H:^{14}C (heavy to light isotope) in products at a fractional conversion, f, and R_∞ is the ratio of ^{3}H:^{14}C in products when the reaction reaches completion.

<u>Additional Results</u>

Like previous studies,[3,4,8,9] our ^{3}H-labeled substrates were all racemic mixtures, which is synthetically more convenient, and has the added advantage that the same reaction yields both a 2˚ KIE and a 1˚ KIE (as we only analyze products and these lead to tritium in different products). For example, in the 2˚ H/T with H-transfer experiments, half of the ^{3}H from the substrate ends up in the product semicarbazone, contributing to the measurement of the 2˚ KIE, but the other half ends up in the product NADH, allowing for the measurement of the 1˚ H/T KIE. Note that since we determine f from the ^{14}C contents of reactants and products, the fact that ^{3}H is split between two products does not affect the calculation of f. Our results for the 1˚ H/T KIE (7.0 ± 0.2) and 2˚ H/T KIE with H-transfer (1.30 ± 0.02) are within error of those previously measured for this enzyme,[3]

which gives us confidence about the validity of both their and our experimental methods. The labeling scheme we used to measure the 2˚ H/T KIE with D-transfer does not yield the 1˚ D/T KIE, but a more complicated KIE that involves differences in both the 1˚ and 2˚ isotopes. Specifically, the 7*R*-[^{2}H]-phenyl-[^{14}C]-benzyl alcohol has D at the 1˚ position and H at the 2˚ position, while the other substrate (one of the enantiomers of the racemic material) has T at the 1˚ position, but D at the 2˚ position. Thus, the measured KIE (1.57 ± 0.08) involves mixed 1˚ and 2˚ effects and we do not speculate on how best to interpret the value; we only point out that the measured value is significantly deflated from the naïve prediction of semiclassical theory that this mixed KIE will be the product of the 1˚ D/T KIE (1.73 ± 0.03)[3] and the 2˚ H/D KIE (1.21 ± 0.01 , from 2˚ H/T KIE and SSE), which is 2.09 ± 0.04.

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