Tritiated Chiral Alkanes as Substrates for Soluble Methane Monooxygenase from *Methylococcus capsulatus* (Bath): Probes for the Mechanism of Hydroxylation

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Abstract: The tritiated chiral alkanes (*S*)-[1-^{2}H$_1$,1-^{3}H]ethane, (*R*)-[1-^{2}H$_1$,1-^{3}H]ethane, (*S*)-[1-^{2}H$_1$,1-^{3}H]butane, (*R*)-[1-^{2}H$_1$,1-^{3}H]butane, (*S*)-[2-^{3}H]butane, (*R*)-[2-^{3}H]butane, and racemic [2-^{3}H]butane were oxidized by soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus* (Bath), and the absolute stereochemistry of the resulting product alcohols was determined in order to probe the mechanism of substrate hydroxylation. When purified hydroxylase, coupling protein, and reductase components were used, the product alcohol displayed 72% retention of stereochemistry at the labeled carbon for the ethane substrates and 77% retention for the butanes labeled at the primary carbon. A putative alkyl radical which would yield these product distributions would have a lifetime of 100 fs, a value too short to correspond to a discrete intermediate. Intramolecular k_H/k_D ratios of 3.4 and 2.2 were determined for ethane and butane, respectively. When the hydroxylations were performed with purified hydroxylase but only a partially purified cellular extract for the coupling and reductase proteins, different product distributions were observed. These apparently anomalous results could be explained by invoking exchange of hydrogen atoms at the α carbon of the product alcohols. The characteristics of this exchange reaction are discussed. Hydroxylation of [2-^{3}H]butanes by the latter system yielded ∼90% retention of stereochemistry at the labeled carbon. The implication of these results for the catalytic mechanism of sMMO is discussed. Together with the mechanistic information available from a range of substrate probes, the results are best accounted for by a nonsynchronous concerted process involving attack on the C−H bond by one or more of several pathways discussed in the text.

Introduction

The methanotrophic bacteria *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b rely on a methane monooxygenase (MMO) system to convert methane into methanol in the first step of their biosynthetic pathways.[1−3] This reaction provides the organisms with their sole source of carbon and energy according to eq 1. MMO exists in both particulate and soluble forms.[4] The membrane-bound form contains copper and is active under normal growth conditions.[5−7] When grown in media deficient in copper, methanotrophs express the soluble MMO system.[8,9] Soluble MMOs from both *Methylococcus capsulatus* (Bath)[10] and *Methylosinus trichosporium* OB3b[11,12] comprise three proteins, an iron-containing hydroxylase component (H) which binds the hydrocarbon substrate and dioxygen,[8] a reductase (R) containing Fe_2S_2 and FAD cofactors which enable it to accept electrons from NADH and transfer them to the hydroxylase,[10,13] and a regulatory or coupling component, protein B.[14,15] In addition to methane, the sMMO system can oxidize a wide variety of substrates.[16−18]

$$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + H_2O + NAD^+ \quad (1)$$

X-ray crystallographic studies of the resting, diferric form of the *M. capsulatus* (Bath) hydroxylase,[19,20] EPR and ENDOR

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spectroscopy of the mixed-valent state,[21−24] and other physical methods[12,22] indicate that the hydroxylase active site contains a non-heme dinuclear iron center.[25] Crystal structures of the protein in several other forms provide clues about the nature of species formed during turnover.[26] In addition, the catalytic cycle has been explored by using freeze-quench and stopped-flow methodology, which allowed intermediate species to be detected.[15,27,28,31] The diferric resting center is reduced by the reductase to give a diferrous center, the structure of which in the absence of the other components is known.[26] This moiety binds dioxygen, forming a diiron(III) peroxo species, which then converts to the active oxidizing intermediate known as compound Q. Intermediates before the peroxo, and after Q, have been postulated but not directly observed for the *M. trichosporium* OB3b enzyme.[29,30] One intermediate termed T, occurring after Q, has been observed only with the substrate nitrobenzene and has been assigned as protein-bound product.[31] The exact formulation of Q has not been elucidated, although it has been suggested to be a high valent iron-oxo species.[2,3] The mechanism by which Q oxidizes substrate is also not known, and some facets of the mechanism have been postulated by analogy to cytochrome P-450.

The heme enzyme cytochrome P-450 displays similar reactivity to sMMO with the exception that it will not hydroxylate methane.[32] Cytochrome P-450 oxidations are thought to proceed through a high valent iron oxo, or ferryl, intermediate.[32−35] This species is postulated to abstract a hydrogen atom from the hydrocarbon substrate, resulting in a substrate radical and an iron-coordinated hydroxyl radical. The hydroxyl radical and substrate radical recombine in a "rebound" reaction to afford the product alcohol, which dissociates from the active site leaving the resting state of the enzyme. Radical clock substrate probes which rearrange upon formation of a substrate radical provided early support for the presence of substrate radicals in the reaction cycle of cytochrome P-450.[36−38]

Recently, some investigators have questioned the validity of the rebound model for cytochrome P-450. In one study employing a very fast radical clock substrate probe, the calculated radical rebound rate was too fast to be rationalized on the basis of a discrete radical intermediate.[39] In the same study, no correlation was observed between the ring opening rate of a range of probes and the amount of ring-opened products observed after oxidation of those probes by cytochrome P-450. Later work employing a probe designed to discriminate between radical and cation intermediates led to the proposal of a nonsynchronous concerted mechanism for the P-450 reaction.[40] A study of this enzyme activated by H_2O_2 concluded that an Fe(III) peroxo, not a ferryl, was responsible for hydroxylation of lauric acid.[41] Theoretical calculations indicate that a more likely scenario than radical rebound, at least for quadricyclane oxidation, might involve HO^+ insertion into a C−C bond rather than radical rebound.[42]

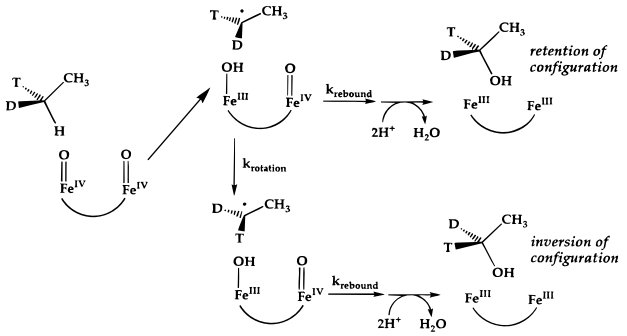

Figure 1. Oxidation of (*S*)-[1-^{2}H$_1$,1-^{3}H]ethane at the C−^{1}H bond via a proposed substrate radical intermediate. Radical rebound before rotation about the C−C bond in the resulting planar intermediate would lead to products displaying retention of configuration, as shown. Rotation before rebound would lead to inverted products.

Radical clock substrate probe studies with sMMO isolated from *M. capsulatus* (Bath) afforded no evidence for the formation of substrate radicals.[43,44] Based on the known rate constants for rearrangement of the various probes, a lower limit of 10^{13} s^{-1} at 45 °C was estimated for a putative rebound reaction rate constant. For enzyme isolated from *M. trichosporium* OB3b, however, evidence for a substrate radical was detected in the form of 3−6% rearranged product alcohols.[43,45] Based on the amount of rearrangement observed, a rate constant of 6 to 9 × 10^{12} s^{-1} at 30 °C was estimated for a putative rebound reaction for this enzyme.[43] In parallel work, information about the nature of possible intermediate substrate radicals was obtained by allowing sMMO from *M. trichosporium* OB3b to hydroxylate both (*R*)- and (*S*)-[1-^{2}H$_1$,1-^{3}H]ethane.[46] As illustrated in Figure 1, if the reaction were to proceed through a radical rebound mechanism, some inversion at the chiral carbon atom would be expected. In the figure, intermediate Q is depicted as a bis(oxo)iron(IV) or diferryl species, although this formulation is only one alternative.[15] Following abstraction of a hydrogen atom from the chiral substrate, an alkyl radical results. This substrate radical can rebound, forming the

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unrearranged product alcohol. Alternatively, because the carbon radical is planar, rotation about the C−C bond might occur prior to recombination, affording a product alcohol with the opposite configuration. The alcohol products were derivatized with a chiral acid, and tritium nuclear magnetic resonance (NMR) analysis of the resulting diastereomeric esters was used to quantitate the amount of inversion that occurred during the hydroxylation reaction. For both (*R*)- and (*S*)-chiral ethane substrates, ≈65% of the product alcohols retained their stereochemistry, whereas ≈35% were inverted.[46] Taking the energy barrier for rotation about the C−C bond to be 0.15 kcal/mol,[47,48] we compute the rate constant for this process to be 4.9×10^{12} s^{-1} at 30 °C (eq 2). From the amount of retention of stereochemistry in the chiral alcohol products, a value of 4.2×10^{12} s^{-1} was calculated for the rebound reaction rate constant (eq 3). This value agrees well with that estimated from the radical clock work.[43]

$$k_{(\text{rotation})} = (kT/h)(\exp(-\Delta G^{\ddagger}/RT)) \quad (2)$$

$$k_{\text{rebound}} = k_{\text{rotation}}(\%\ \text{retention}/\%\ \text{inversion} - 1) \quad (3)$$

In view of the slight differences in reactivity toward radical clock substrate probes exhibited by sMMO from the different organisms, we were interested to determine how the *M. capsulatus* (Bath) enzyme would perform in the chiral ethane experiment.[46] Accordingly, the substrates (*S*)-[1-^{2}H$_1$,1-^{3}H]-ethane, (*R*)-[1-^{2}H$_1$,1-^{3}H]ethane, (*S*)-[1-^{2}H$_1$,1-^{3}H]butane, (*R*)-[1-^{2}H$_1$,1-^{3}H]butane, (*S*)-[2-^{3}H]butane, (*R*)-[2-^{3}H]butane, and racemic [2-^{3}H]butane were hydroxylated with sMMO from *M. capsulatus* (Bath), the results and analysis of which are reported here.

Experimental Section

Bacterial Growth and Protein Purification. *Methylococcus capsulatus* (Bath) cells were grown as described previously.[49] Hydroxylase and recombinant protein B were purified as reported elsewhere, and the iron content for hydroxylase and specific activities were in the usual ranges.[22,43] In some reactions, an unresolved mixture of protein B, reductase, and other cellular proteins (B/R mix) from the initial DEAE cellulose column (0.05−0.5 M NaCl fraction) was used with purified hydroxylase to reconstitute the enzymatic system.[18] In all other experiments, purified recombinant B[22,43] and purified native reductase (vide infra) were employed.

Isolation of sMMO Reductase from Soluble Cell Extract. Protein purification procedures were performed at 4 °C or on ice. Cell lysis with a French pressure cell (American Instrument Co.) and the preparation of soluble cell extract were carried out as previously described[43] except for the buffer composition, which followed a published procedure.[50] Frozen cells (∼100 g) were suspended in 200 mL of 25 mM *N*-morpholinopropane sulfonic acid (MOPS) buffer (pH 7.0) which contained 200 μM of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 2 mM cysteine (buffer A) as well as 0.01 mg/mL of DNase 1 and 2 mM phenylmethylsulfonyl fluoride (PMSF). Buffers containing iron and cysteine were prepared just prior to use. After the cells were lysed, sodium thioglycolate (NaTG) was added to a final concentration of 8 mM, which was maintained in all subsequent steps of the reductase purification. The lysed cells were centrifuged in a Beckman L-70 ultracentrifuge at 32 000 × g for 10 min. The supernatant was centrifuged at 90 000 × g for 1 h. An additional 10 min centrifugation (90 000 × g) of the supernatant removed residual particles, allowing the supernatant to be filtered with a 0.2 μm syringe filter. The soluble cell-free extract was diluted with an equal volume of buffer A, and the sMMO protein components were separated on a DEAE Sepharose fast flow (Pharmacia LKB Biotechnology Inc.) column which was packed (5.0 × 22 cm) and equilibrated in buffer A by using a Pharmacia FPLC chromatographic system. All buffers were filtered and degassed prior to use. The sample was loaded at approximately 4 mL/min by using an in-line peristaltic pump. The loaded sample was washed at the same flow rate with 800 mL of buffer A. An eight column volume gradient of 0−0.4 M NaCl in buffer A was employed to elute bound protein. The flow rate applied during the salt gradient was 3.5 mL/min. Native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE analysis were used to evaluate the protein fractions.

Fractions which contained protein of the correct molecular weight according to SDS-PAGE analysis, and which reduced dichlorophenol indophenol at a rate which was greater than 30% of the most active fraction,[51] were pooled as crude reductase protein. The crude reductase sample was diluted with an equal volume of 20 mM Tris (pH 7.1) with 8 mM NaTG (Tris buffer) and 50 mM NaCl and was loaded at a flow rate of 3 mL/min on a DEAE Sepharose CL-6B column (2.6 × 8.0 cm). The loaded sample was washed with 2.8 column volumes of Tris buffer with 100 mM NaCl. The bound protein was eluted with a 10 column volume gradient of Tris buffer from 100−500 mM NaCl. Fractions from DEAE Sepharose CL-6B chromatography were pooled which had an A_{458nm}/A_{340nm} ratio of greater than 1.0. The pooled material was applied to a 5′AMP Sepharose 4B affinity (Pharmacia) column (2.5 × 11 cm). The affinity column had been regenerated and equilibrated in Tris buffer, 50 mM NaCl.

The loaded sample was washed with 1.3 column volumes of Tris buffer, 95 mM NaCl. This wash eluted nonadsorbed proteins from the column. One column volume of Tris buffer, 410 mM NaCl followed by a three column volume rinse of Tris buffer, 50 mM NaCl eluted nonspecifically bound protein. Reductase fractions were eluted with Tris buffer and 50 mM NaCl containing 1.4 mM ethanol-free NADH. SDS-PAGE analysis was performed to confirm the absence of any contaminating protein in the purified reductase. The NADH was removed from purified reductase by concentrating the sample to ∼10% of the original volume with an Amicon concentrating cell equipped with an ultrafiltration PM30 membrane, which was run under 30 psi of N_2 pressure. The reductase was rediluted in Tris buffer containing 50 mM NaCl and reconcentrated. This procedure was repeated until no NADH was detected spectrophotometrically in the eluate. A typical yield of 20−30 mg of purified reductase was obtained from 100 g of cell paste, and specific activities were in the range of 1200−1600 mU/mg under the conditions necessary to generate a high product concentration (vide infra). In a standard propylene assay, activities ranged from 3500−3800 mU/mg.[22,43]

Determination of Conditions for Oxidizing Labeled Alkanes. Preliminary assays with unlabeled ethane, butane, and propylene were carried out to determine conditions which afforded 1−2 μmol of product alcohol. Hydroxylase was concentrated to ≈300 μM and incubated with the B/R mixture or the purified components in varying ratios in a 5 mL reaction flask capped with a septum. The total volume was adjusted to 400 μL with 25 mM MOPS, pH 7.0 buffer. A syringe was inserted through the septum, and 2 mL of the head space gas was removed and replaced with 2 mL of the substrate gas. The mixture was incubated for 30 s at 45 °C in a shaking incubator, after which time 100 μL of a 0.1 M ethanol-free solution of NADH was added to initiate the reaction. The flask was returned to the incubator, and the reaction was allowed to proceed for 5 min. A 5 μL portion of the solution was injected into a gas chromatograph equipped with a Porapak Q column to quantitate the alcohol product present in the reaction solution.[43] The area of the product peak was compared to a calibration curve of alcohol peak area versus the concentration of sample injected. Conditions which afforded at least 1 μmol of alcohol product per 1 mL of reaction volume were subsequently employed at the National Tritium Labelling Facility. Typically 50−80 nmol each of H and B and 42−68 nmol of R were employed. Under these conditions, the specific activity calculated for the hydroxylase is low, ≈40 mU/mg, owing to product inhibition.

Reagent Synthesis. Materials and General Procedures. ^{1}H, ^{2}H, and ^{13}C NMR spectra were recorded on either a Bruker AC-200 or AF-300 spectrometer. Chemical shifts were determined relative to the

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residual proton or ^{13}C absorption of the solvent, $CDCl_3$ or C_6D_6, used as internal standard. ^3H spectra were recorded at 320.13 MHz by using an IBM-Bruker AF-300 spectrometer fitted with a tritium channel and a dual $^1H/^3H$ 5 mm probe. Stable isotope compounds were obtained from Aldrich Chemical Co. or Cambridge Isotope Labs. (*R*)-*O*-Acetylmandelic acid and (*S*)- and (*R*)-2-butanol were purchased from Aldrich Chemical Co. and were of 99% ee. THF was dried by distillation from Na/benzophenone ketyl, CH_2Cl_2 by distillation from CaH_2, and pyridine by storing over KOH pellets. All other solvents were of HPLC grade and used without further purification. Tosyl chloride was recrystallized from hexane and dried in vacuo overnight prior to use. Argon was dried by passing through a bed of 3 Å molecular sieves and then anhydrous $CaCO_3$. Flash chromatography was performed over silica gel 60 (230−400 mesh).

(*S*)-[1-$^2H_1$]Ethanol. [1-$^2H_1$]Acetaldehyde (0.56 mL, 10 mmol) (Cambridge Isotope Labs) was added to a stirred solution of (*R*)-Alpine Borane (10 mmol) in 20 mL of dry tetrahydrofuran (THF) at −78 °C under argon. The temperature was maintained at −78 °C for 4 h, after which time the mixture was allowed to warm to room temperature. After 15−18 h, the solution was cooled to 0 °C, and ethanolamine (0.74 mL, 12.3 mmol) was added. After 30 min a white precipitate formed. The solvent was removed by distillation, during which the precipitate dissolved upon warming. Just before the end of the distillation, 5 mL of benzene was added to, and distilled from, the mixture. The benzene addition and distillation steps were repeated twice. The final volume of the collected distillates was 32 mL. (*R*)-[1-$^2H_1$]Ethanol was synthesized from (*S*)-Alpine Borane by using similar procedures.

(*S*)-[1-^{2}H]Ethyl Tosylate. To a solution of tosyl chloride (3.5 g, 18.4 mmol) in dry pyridine (15 mL) was added (*S*)-[1-^{2}H]ethanol (2 g, 43.4 mmol). A crystalline precipitate of pyridine hydrochloride was visible after 5 min, and the resulting suspension was stirred under argon for 5 h at room temperature. Reaction was terminated by addition of diethyl ether (50 mL) and washed with saturated aqueous $CuSO_4$ (1 × 50, 2 × 25 mL), 1 N aqueous HCl (25 mL), and saturated aqueous $NaHCO_3$ (25 mL), dried over $MgSO_4$ and then filtered. The solvent was removed in vacuo, and the title compound recovered as a white crystalline solid by chromatography over flash silica gel eluting with 10% diethyl ether in hexane (2.42 g, 68%, mp 37 °C). ^1H NMR: δ_H (300 MHz, $CDCl_3$): 1.22 (3H, t, 7.1 Hz, H-2), 2.39 (3H, s, Ar-Me), 4.06 (2H, q, 7.1 Hz, H-1), 7.30 (2H, d, 8.2 Hz, Ar), 7.73 (2H, d, 8.2 Hz, Ar) ppm. ^2H NMR: (46.1 MHz, C_6H_6) 3.74. (*R*)-[1-^{2}H]Ethanol was used to prepare the (*R*)-[1-^{2}H]ethyl tosylate.

Ethyl (2′*R*)-2′-Acetoxy-2′-phenylethanoate. To a solution of (*R*)-O-acetylmandelic acid (830 mg, 4.3 mmol) and 4-(dimethylamino)-pyridine (DMAP) (16 mg) in dry methylene chloride (11 mL) at −20/−30 °C was added, dropwise over 10 min, a solution of dicyclohexylcarbodiimide (DCC) (880 mg, 4.3 mmol) in dry methylene chloride (5 mL). After 10 min a pale cream precipitate was observed. Ethanol (500 mg 10.9 mmol) was added dropwise with the temperature maintained between −20/−30 °C, the solution was stirred for 90 min, and then the temperature was allowed to rise to ambient conditions overnight.

The resulting suspension was filtered, and the solids were washed with methylene chloride (3 × 15 mL). The mixture was washed with brine (40 mL), saturated aqueous $NaHCO_3$ (35 mL), the organics were dried over $MgSO_4$, and the solvent was removed in vacuo to leave a yellow mass. This product was purified by chromatography over flash silica gel eluting with ethyl acetate/hexane (10:70) to yield the title compound as a colorless oil (955 mg, 67%). ^1H NMR: δ_H (300 MHz, $CDCl_3$): 1.19 (3H, t, 7.1 Hz, H-2), 2.16 (3H, s, $COCH_3$), 4.14 (2H, m, H-2), 5.89 (1H, s, H-2′), 7.35 (3H, m, Ar) and 7.45 (2H, m, Ar) ppm.

[1-$^2H_2$]Butan-1-ol. To a suspension of $LiAlD_4$ (15.4 g, 0.37 mol) in anhydrous THF (120 mL) under argon at room temperature was added methyl butyrate (61 g, 0.61 mol) at a rate sufficient to initiate, and then maintain, gentle reflux over approximately 25 min. The resulting slurry was stirred under reflux for 3 h and then overnight at room temperature. The resulting mixture was quenched by addition of water (10 mL) followed by diethyl ether (100 mL) and after 15 min 1 N aqueous HCl (125 mL). The organic fraction was separated, and the aqueous was extracted with diethyl ether (4 × 50 mL). The combined organic layers were dried over anhydrous $MgSO_4$ for 1 h and then filtered. The resulting solution was fractionally distilled at atmospheric pressure to yield the title compound as a colorless oil (20.4 g, 45%). ^1H NMR: δ_H (200 MHz, $CDCl_3$): 0.29 (3H, t, 7.3 Hz, H-4), 1.27 (2H, sext, 7.3 Hz, H-3), 1.43 (2H, t, 7.3 Hz, H-2), 3.23 (1H, bs, OH) ppm. ^2H NMR: δ_D (46.1 MHz, C_6H_6): 3.34 (s) ppm.

[1-$^2H_1$]Butyraldehyde. [1-$^2H_2$]Butan-1-ol (20 g, 0.26 mol) was heated to a gentle reflux, and a solution of $K_2Cr_2O_7$ (28.3 g, 0.1 mol) in concentrated H_2SO_4 (20 mL) was then added via a nonpressure equalizing dropper funnel, at such a rate so as to maintain a constant distillation of the product [1-$^2H_1$]butyraldehyde as a water azeotrope. After addition of all the dichromate solution the mixture was heated at reflux for a further 10 min (maximum stillhead temperature 89 °C). The resulting aqueous layer was discarded, and the organic layer was dried over $MgSO_4$ (2 g) for 30 min and then purified by fractional distillation. The fractions which distilled between 50 and 75 °C were pooled and again fractionally distilled; fractions were collected with stable stillhead temperatures of 67 °C and 70 °C. NMR analysis showed no trace of codistilling 1-[1-$^2H_2$]butanol, but fraction 1 contained a small quantity (<3%) of the corresponding hemiacetal. Combined yield = 6.2 g, 85.5 mmol, 33%. ^1H NMR: δ_H (200 MHz, $CDCl_3$): 0.88 (3H, t, 7.4 Hz, H-4) ppm, 1.59 (2H, sext, 7.3 Hz, H-3), 2.33 (2H, t, 7.2 Hz, H-2). ^2H NMR: δ_D (46.1 MHz, C_6H_6): 9.25 (s) ppm. ^{13}C NMR: δ_C (75.5 MHz, C_6D_6): 13.60, 15.55, 45.38, and 202.62 (t, $J_{D-^{13}C}$ = 25.9 Hz) ppm.

(*R*)-1-[1-$^2H_1$]Butanol. A solution of (*S*)-Alpine Borane (25 mmol) in anhydrous THF (50 mL) was cooled to −78 °C under argon and stirred for 1 h. [1-$^2H_1$]Butyraldehyde (2 mL, 22 mmol) was added dropwise, and the resulting solution stirred at −78 °C for 2 h and then at room temperature overnight. The resulting solution was cooled to 0 °C, ethanolamine (2 mL, 32.5 mmol) was added dropwise, and the solution was stirred at 0 °C for 30 min and then at room temperature overnight. The resulting solution was fractionally distilled to remove all volatiles boiling below 75 °C; xylenes (30 mL) were added, and the solution again distilled with all volatiles boiling between 75−130 °C collected (19.3 mL). (*S*)-1-[1-$^2H_1$]Butanol was prepared analogously from (*R*)-Alpine Borane.

(*R*)-1-[1-$^2H_1$]Butyl Tosylate. To a solution of tosyl chloride (3.8 g, 20 mmol) in dry pyridine (20 mL) was added the (*R*)-butanol/xylenes mixture (15.3 mL, max: 17.6 mmol). A crystalline precipitate of pyridinium hydrochloride was visible after 5 min, and the resulting lemon yellow solution was stirred under argon for 5 h at room temperature. The reaction was terminated by addition of diethyl ether (100 mL) and washed with saturated aqueous $CuSO_4$ (1 × 100, 2 × 50 mL), 1 N aqueous HCl (50 mL), and saturated aqueous $NaHCO_3$ (50 mL), dried over anhydrous $MgSO_4$, and then filtered. The solvent was removed in vacuo and the (*R*)-1-[1-$^2H_1$]butyl tosylate recovered as a colorless oil by chromatography over flash silica gel eluting with 7.5% diethyl ether in hexanes (2.43 g, 60%). ^1H NMR: δ_H (200 MHz, $CDCl_3$): 0.87 (3H, t, 7 Hz, H-4), 1.34 (2H, sext, 8.1 Hz, H-3), 1.62 (2H, q, 7.5 Hz, H-2), 2.45 (3H, s, Me), 4.02 (1H, m, H-1), 7.34 (2H, d, 8.3 Hz), 7.78 (2H, dd, 8.3 and 1.8 Hz) ppm. ^2H NMR: δ_D (46.1 MHz, C_6H_6): 3.79 (s). ^{13}C NMR: δ_C (75.5 MHz, $CDCl_3$): 13.31, 18.50, 21.55, 30.61, 70.03 (t, 22.9 Hz), 127.79, 129.75, 133.17, and 144.59 ppm. (*S*)-1-[1-$^2H_1$]butyl tosylate was prepared analogously from (*S*)-butanol.

(1R)-[1-$^2H_1$]Butyl (2′R)-2′-Acetoxy-2′-phenylethanoate. To a solution of (*R*)-*O*-acetylmandelic acid (1.20 g, 6.1 mmol) and DMAP (16 mg) in dry methylene chloride (25 mL) at −20/−30 °C was added, dropwise over 10 min, a solution of DCC (1.40 g, 6.4 mmol) in dry methylene chloride (10 mL) to give a pale cream precipitate. A fraction of the (*R*)-1-[1-$^2H_1$]butanol/xylenes mixture (4 mL) was added dropwise with the temperature maintained between −20/−30 °C and stirred for 90 min, and the solution was allowed to reach room temperature overnight. The resulting cream suspension was filtered, and the filtrate was washed with saturated aqueous $NaHCO_3$ (2 × 50 mL) and dried over $MgSO_4$, and the solvent was removed in vacuo to leave a yellow mass. The title compound was recovered by chromatography over flash silica gel eluting with 10% ethyl acetate in hexane and analyzed by ^2H NMR to establish the optical purity of the sample; ee = 93(±3)%. ^2H NMR: δ_D (46.1 MHz, C_6D_6): 3.89 (s, D-1, major isomer), 3.82 (s, D-1, minor isomer) ppm. The other enantiomer was prepared similarly from (*S*)-1-[1-$^2H_1$]butanol and displayed 95% ee.

(±)-2-Butyl Tosylate. To a solution of tosyl chloride (1.05 g, 5.5 mmol) in dry pyridine (10 mL) was added (±)-2-butanol (1.20 mL, 13

mmol). A crystalline precipitate of pyridinium hydrochloride was visible after 5 min, and the resulting solution was stirred under argon for 5 h at room temperature. The reaction was terminated by addition of diethyl ether (50 mL) and washed with saturated aqueous $CuSO_4$ (1 × 50 mL, 2 × 25 mL), 1 N aqueous HCl (30 mL), and saturated aqueous $NaHCO_3$ (30 mL), dried over $MgSO_4$, and then filtered. The solvent was removed in vacuo, and the title compound was recovered as a colorless oil by chromatography over flash silica gel eluting with 7.5% diethyl ether in hexanes (799 mg, 64%). (*S*)-2-Butyl tosylate and (*R*)-2-butyl tosylate were prepared analogously from (*S*)-2-butanol and (*R*)-2-butanol, respectively. ^{1}H NMR: δ_H (300 MHz, $CDCl_3$): 0.78 (3H, t, 7.9 Hz, H-4), 1.21 (3H, d, 6.3 Hz, H-1), 1.54 (2H, m, H-3), 2.40 (3H, s, Me-4′), 4.52 (1H, tq, H-2), 7.29 (2H, d, 7.9 Hz, H-2′ and H-6′), 7.75 (2H, d, 6.6 Hz, H-3′ and H-5′) ppm.

(2S)-Butyl (2′R)-2′-Acetoxy-2′-phenylethanoate. To a solution of (*R*)-*O*-acetylmandelic acid (829 mg, 4.26 mmol) and DMAP (14 mg) in dry methylene chloride (11 mL) at −20/−30 °C (acetone/dry ice) was added, dropwise over 10 min, a solution of DCC (880 mg, 4.27 mmol) in dry methylene chloride (4 mL). After 10 min a pale cream precipitate was observed. (*S*)-2-Butanol (300 μL, 3.25 mmol) was added dropwise with the temperature maintained between −20/−30 °C and stirred for 90 min, and then the solution was allowed to reach room temperature over a further 90 min. The resulting suspension was filtered, and the solids were washed with methylene chloride (3 × 15 mL). The mixture was washed with brine (40 mL) and saturated aqueous $NaHCO_3$ (35 mL), the combined organics were dried over $MgSO_4$ and the solvent was removed in vacuo to leave a yellow/white mass. This product was purified by chromatography over flash silica gel (45 g) eluting with ethyl acetate/hexane (5:70) to yield the title compound as a colorless oil (678 mg, 89%). ^{1}H NMR: δ_H (500 MHz, C_6D_6): 0.75 (3H, t, 7.4 Hz, H-4), 0.86 (3H, d, 6.3 Hz, H-1), 1.25 (1H, pseudo sept, 7.2 Hz, H-3R), 1.42 (1H, pseudo sept, 7.2 Hz, H-3S), 1.73 (3H, s, $COCH_3$), 4.86 (1H, m, H-2, 6.03 (1H, s, H-2′), 7.42 (3H, m), 7.48 (2H, m). Selective decoupling at 0.75 ppm: 1.24 (1H, dd, 13.8 and 5.5 Hz, H-3R), 1.42 (13.7 and 7.2 Hz, H-3S) ppm.

(2R)-Butyl (2′R)-2′-Acetoxy-2′-phenylethanoate. The experimental protocol for the (2S)-isomer was used, except that R-2-butanol was employed. ^{1}H NMR: δ_H (500 MHz, C_6D_6): 0.46 (3H, t, 7.5 Hz, H-4), 1.03 (3H, d, 6.2 Hz, H-1), 1.10 (1H, pseudo sept, 7.3 Hz, H-3S), 1.24 (1H, pseudo sept, 7.3 Hz, H-3R), 1.74 (3H, s, $COCH_3$), 4.82 (1H, m, H-2), 6.03 (1H, s, H-2′), 7.42 (3H, m), 7.48 (2H, m). Selective decoupling at 0.46 ppm: 1.10 (1H, dd, 16.1 and 5.1 Hz, H-3S), 1.24 (1H, dd, 16.2 and 8.4 Hz, H-3R) ppm.

***n*-Butyl (2′R)-2′-Acetoxy-2′-phenylethanoate.** The experimental protocol for the (2S)-isomer was employed, except that 1-butanol was used. ^{1}H NMR: δ_H (500 MHz, C_6D_6): 0.61 (3H, t, 7.4 Hz, H-4), 0.99 (2H, pseudo sept, 7.4 Hz, H-3), 1.20 (2H, dquin, 6.8 and 2.0 Hz, H-2), 1.73 (3H, s, $COCH_3$), 3.85 (1H, m, H-1S), 3.94 (1H, m, H-1R), 6.06 (1H, s, H-2′), 7.40 (3H, m) and 7.45 (2H, m) ppm.

Verification of Butyl and Ethyl Tosylate Stereochemistry. To a mixture of (*R*)-*O*-acetylmandelic acid (42 mg, 0.2 mmol) and anhydrous K_2CO_3 (28 mg, 0.2 mmol) in dry dimethylformamide (DMF) (1 mL) stirring vigorously at room temperature under argon was added a solution of the alkyl tosylate (0.2 mmol) in dry DMF (0.5 mL). After 30 min a fine precipitate of tosic acid had formed. After 36 h the solvent was removed in vacuo, and the flask was pumped under high vacuum overnight. The residual gum was washed with benzene (5 × 3 mL), and the combined fractions were filtered through a plug of flash silica gel (1 cm, Pasteur pipette) and eluted with benzene (10 mL). The benzene was removed in vacuo, and the product was analyzed by ^{2}H NMR.

(*S*)-[2-^{2}H$_1$, ^{3}H]Acetate. A sample of the derivative product resulting from oxidation of (*S*)-[2-^{2}H$_1$, ^{3}H]ethane was stirred in toluene (200 μL) and 2 N KOH (600 μL) at room temperature for 4 h. Carrier ethanol (10 μL) was added, and the volatiles were isolated by lyophilization. The isolated volatiles were added to a stirred solution of $KMnO_4$ (160 mg) and K_2CO_3 (120 mg) in water (500 μL) at 4 °C. The resulting mixture was brought to room temperature and stirred for 6 h. The brown suspension was acidified to pH ≈ 1 with 6 M H_2SO_4, and the volatiles were isolated by lyophilization. The recovered volatiles were adjusted to pH 10 with 2 N KOH, and the volatiles were removed by lyophilization. The remaining solid was dissolved in distilled water (1 mL), and a portion (*ca.* 5 × 10^4 cpm) was analyzed for chiral purity.[52] The compound 2R was treated similarly.

Chiral Tritiated Alkanes. The reagent $LiEt_3B^3H$ was prepared as described previously.[53] The appropriate alkyl tosylate (≈0.3 mmol), dissolved in 100 μL of THF, was added to a round bottom flask equipped with a stopcock connected to a closed vacuum system and containing ≈0.2 mmol of $LiEt_3B^3H$ in THF. Vigorous gas evolution occurred for 15−20 s. After a 1 h incubation period, the flask was cooled to −78 °C. The gas was transferred to a coconut charcoal bed (200 mg) in a test tube immersed in liquid nitrogen. The transfer was allowed to occur for 60 s. Identical procedures were used to synthesize (*S*)-[1-^{2}H$_1$,1-^{3}H]ethane, (*R*)-[1-^{2}H$_1$,1-^{3}H]ethane, (*S*)-[1-^{2}H$_1$,1-^{3}H]butane, (*R*)-1-[^{2}H$_1$,1-^{3}H]butane, (*S*)-[2-^{3}H]butane, (*R*)-[2-^{3}H]butane, and racemic [2-^{3}H]butane from their corresponding alkyl tosylates.

Enzymatic Reactions. A second round bottom flask equipped with a stopcock, sidearm, and septum was fitted to the reaction apparatus described above and evacuated. Buffer (25 mM MOPS, pH 7.0, 100−500 μL) was added through the septum and cooled to 77 K. Substrate gas was transferred to the flask from the charcoal bed by opening both stopcocks and bringing the charcoal bed to room temperature by warming with hot air. After 60 s the stopcocks were closed, and the buffer was brought to room temperature. The protein solution was added through the septum. A quantity of NADH (50 or 100 μL of a 0.1 M solution) was also added. Pure O_2 gas (1 mL) was added by syringe through the septum], and the mixture was incubated at 45 ± 2 °C with constant stirring for 30 min. Cooling the flask to −78 °C terminated the reaction. Excess substrate gas was transferred from the reaction flask back to the charcoal bed by first cooling the bed in liquid nitrogen and then opening the stopcocks. Transfer was allowed to proceed for 60 s, and then the stopcocks were closed. A 10 μL aliquot of the expected product alcohol (unlabeled) was added to the reaction flask to act as a carrier in subsequent manipulations. This flask was removed from the apparatus and transferred to a vacuum line where the volatile reaction products were collected by lyophilization.

Product Analysis. To measure the radioactivity of the samples, a 1 μL aliquot of the product solution was diluted in 200 μL of methanol before being placed in a liquid scintillation counter. NMR analysis was carried out with a 300 MHz Bruker spectrometer at NTLF. All ^{3}H NMR spectra were ^{1}H decoupled.

Derivatization.[54] (*R*)-*O*-Acetylmandelic acid (47 mg, 0.24 mmol) and DMAP (2 mg) were mixed in 2 mL of methylene chloride at −40 °C in a 100 mL round bottom flask. Over a 5 min period, a solution of DCC (54 mg, 0.24 mmol) in 0.5 mL of methylene chloride was added dropwise to the flask. A creamy precipitate resulted after 10 min. The enzymatic lyophilyzate was added dropwise over a 5 min period. The resulting mixture was allowed to warm to room temperature over 3−6 h and then stirred overnight. The suspension was filtered through a silica plug (≈2 g) and washed with 35−40 mL of methylene chloride. The filtrate was evaporated to near dryness under a dinitrogen stream and then lyophilized to remove any methylene chloride. The residue was suspended in benzene-d_6 and filtered through a glass wool plug directly into an NMR tube.

Oxidation of C_2D_6. Perdeuteroethane (Cambridge Isotope Labs, 99%) was oxidized by a reconstituted sMMO system as reported above for unlabeled substrates except that the incubation time was 30 min. The product alcohol was isolated from the aqueous reaction mixture by extraction with 1 mL of methylene chloride and the extract was dried over $MgSO_4$. A 1 μL aliquot of distilled pyridine and 1 μL benzoyl chloride were added. The esterification reaction was allowed to proceed for 1 h at room temperature, and the mixture was analyzed by gas chromatography with mass spectrometric detection (GC/MS) on a Hewlett-Packard 5890A gas chromatograph equipped with a Hewlett Packard 5971A mass spectrometer. An HP-1 (methyl silicone gum) column (50 m × 0.2 mm × 0.5 μm film thickness) was employed for the separation.

Exchange Reactions. A 2 μL sample of CD_3CD_2OH or CH_3CD_2-OH (Cambridge Isotope Labs, 99%) was added to 0.9 mL of B/R mix, a reconstituted system consisting only of purified H, B, and R components, or to buffer (25 mM MOPS, pH 7). A 100 μL portion of 0.1 M NADH was added. In some experiments, 1 μL of 0.1 M NAD^+ was also added. The mixture was incubated at 45 °C with shaking for 30 min. The ethanol was isolated as described above and analyzed by GC/MS.

Results

Synthesis of Chiral Tritiated Alkanes. The method for preparing chiral tritiated alkanes applied known and unambiguous chemistry developed for the synthesis of chiral methyl tritiated acetates in high yield, activity, and enantiomeric purity. [1-$^2H_1$]Acetaldehyde was reduced to (*S*)- or (*R*)-[1-$^2H_1$]ethanol by (*R*)- or (*S*)-Alpine Borane, respectively. The ethanols were isolated by distillation and converted to their tosylates by treatment with tosyl chloride in pyridine. An aliquot was also converted to the (*R*)-*O*-acetylmandelic acid ester derivative in the presence of DCC-DMAP. ^2H NMR analysis of the products demonstrated that the alcohols had been synthesized in 88 ± 4% ee. Experiments with standard materials were carried out to assure that the C-1 center in the alcohol does not racemize when this reaction is carried out below −10 °C.[55] Treatment of the ethyl tosylates with (*R*)-*O*-acetylmandelic acid and K_2CO_3 in DMF converted them to their mandelate derivatives with inversion at C-1. ^2H NMR experiments again demonstrated that the ethanols had been prepared in 88 ± 4% ee and confirmed the stereochemical fidelity of the derivatization procedure. For the final generation of chiral tritiated ethane, the tosylate group was converted to tritide by using the reagent $LiEt_3B^3H$ at maximum specific activity.[53] This reaction proceeds via a direct SN_2 displacement, affording ethane of unambiguous stereochemistry.

Primary tritiated butanes were generated in a similar manner. The [1-$^2H_1$]butyraldehyde was prepared by $LiAl^2H_4$ (>98 atom % ^2H) treatment of methyl butyrate to produce [1-$^2H_2$]butanol, which was converted to the aldehyde by treatment with $K_2Cr_2O_7$ in H_2SO_4. This method takes advantage of the primary KIE for this process, yielding product with enriched deuterium content. No [1-$^1H_2$]butanol species were formed, as verified by ^{13}C NMR spectroscopy. Butanes carrying tritium in the secondary position were prepared initially from (*S*)- and (*R*)-2-butanol of >99% ee.

Analysis of Lyophilyzates. For each alkane enantiomer, hydroxylation can take place either at the hydrogen atom or deuterium atom of the chiral carbon, at the tritium atom, or at an unlabeled carbon. Products that arise from hydroxylation of the C−T bond are not detected, because ^3H NMR spectroscopy is the analytical method. Intramolecular kinetic isotope effects, k_H/k_D, were calculated from the relative amounts of C−H versus C−D hydroxylation products. The ^3H NMR data also afforded information about the distribution of hydroxylation at each carbon atom of the substrate. These ratios of products, C2/C1 and C4/C1, for ethane and butane, respectively, were also determined. In alkanes labeled at the primary position, the tritiated carbon is designated as C1. In the butanes labeled at a secondary carbon, that carbon is referred to as C2.

The possible alcohol products of the reaction of (*S*)-[1-$^2H_1$,1-^3H]ethane with sMMO are depicted in Figure 2, and the observed ^3H NMR spectra are given in Figure 3. The ^3H NMR spectrum of the reaction products in Figure 3A contains four major resonances at $\delta = 1.19$ (s), $\delta = 3.65$ (s), $\delta = 3.67$ (s), and $\delta = 4.84$ (s) ppm. The resonance at $\delta = 4.84$ ppm was assigned to ^3HHO, and the singlet at $\delta = 1.19$ ppm to the product of oxidation occurring at the unlabeled carbon atom. The inset in Figure 3 shows an expansion of the two signals at $\delta = 3.65$ and 3.67 ppm. The narrower, more downfield resonance is attributed to the products of oxidation at the C−D bond (RCHTOH products, where R = CH_3), whereas the broader, more upfield signal is assigned as arising from oxidation at the C−H bond (RCDTOH products). The splitting

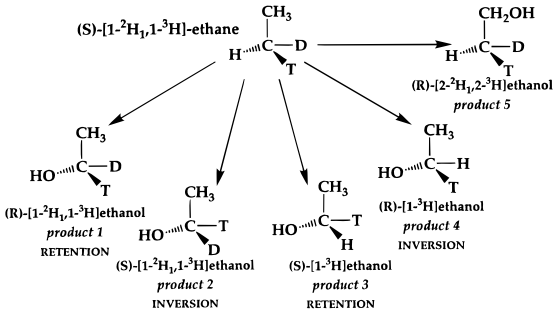

Figure 2. Possible reaction products from the oxidation of (*S*)-[1-$^2H_1$,1-^3H]ethane. Products 1 and 2 arise from reaction at the C−H bond, products 3 and 4 from reaction at the C−D bond, and product 5 from reaction at the unlabeled carbon. Alcohols arising from hydroxylation of the C-T bond are not detected by ^3H-NMR spectroscopy. The ratio of the sum of products 1 and 2 to the sum of products 3 and 4 yields the intramolecular k_H/k_D isotope effect. After derivatization, the products displaying retention of stereochemistry (1 and 3) can be distinguished from those exhibiting inversion (2 and 4), and the total percent retention can be determined.

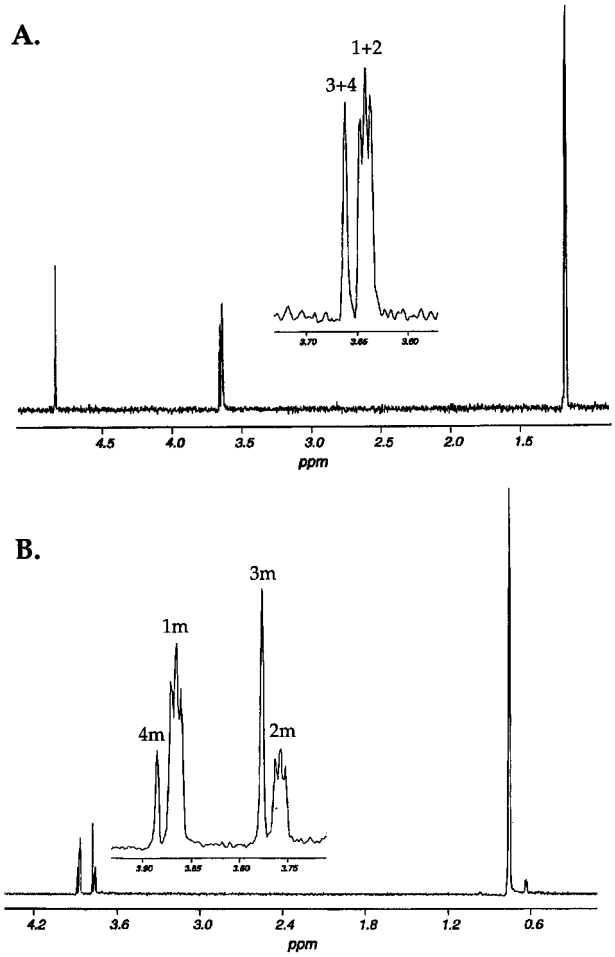

Figure 3. ^3H NMR analyses of the oxidation products of (*S*)-[1-$^2H_1$,1-^3H]ethane. (A) Spectrum of the underivatized alcohols. The far upfield peak corresponds to oxidation at the unlabeled carbon (product 5 from Figure 2). The narrower downfield peak at ~3.65 ppm (inset) arises from alcohols 3 and 4 from Figure 2. The upfield peak, split by the integer-spin deuterium atom, originates from alcohols 1 and 2. The far downfield peak is assigned to tritiated water. (B) Spectrum of the mandelate derivatives. Resonances 1−4 (inset) correspond to the mandelates of the like-numbered products in Figure 2. The large upfield peak results from the mandelate of product 5.

of the latter signal is due to coupling to the deuterium atom present in the molecule. A kinetic isotope effect can be calculated from the ratio of alcohol products for hydroxylation

(52) Floss, H. G.; Tsai, M. D. *Adv. Enzymol.* **1979**, *50*, 243−302.
(53) Andres, H.; Morimoto, H.; Williams, P. G. *J. Chem. Soc., Chem. Commun.* **1990**, 627−628.

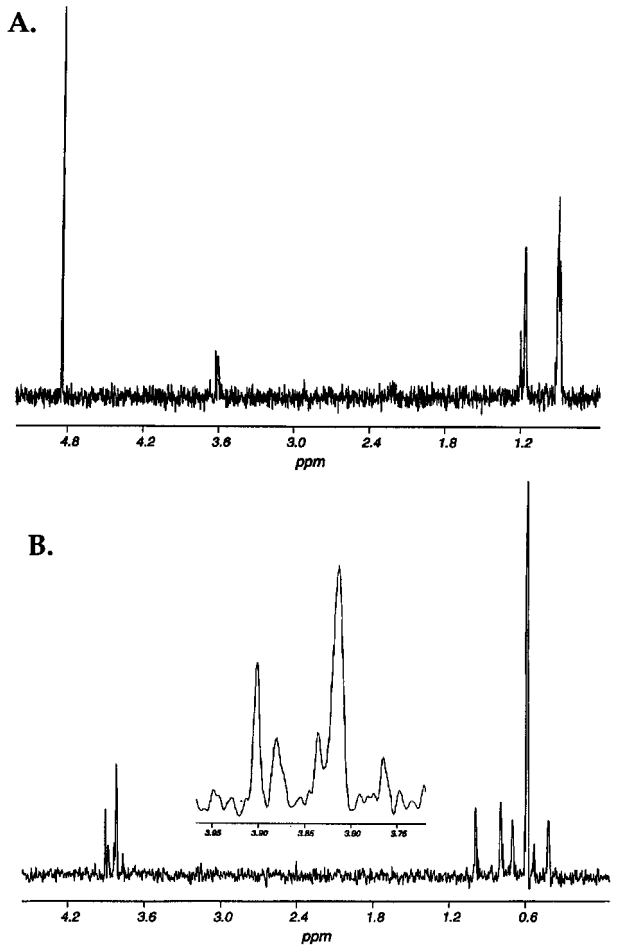

Figure 4. ^{3}H NMR analyses of the oxidation products of (*S*)-[1-^{3}H,1-^{2}H]butane. (A) Spectrum of the alcohol products. Tritiated water appears far downfield. The signal at $\delta = 3.7$ ppm corresponds to the primary alcohols $CH_3CH_2CH_2CHT(OH)$ and $CH_3CH_2CH_2CDT(OH)$. Other signals are identified in the text. (B) The spectra of corresponding mandelate derivatives. The inset is an expansion of the region of the diastereomeric esters of the products arising from oxidation at the labeled carbon.

of the C−H bond versus products hydroxylated at the C−D bond. The sum of the areas of these two peaks can be compared to the area at $\delta = 1.19$ ppm to obtain information about the relative hydroxylation rates at the two different carbon atoms. This value may reflect the relative reactivities of the C−H versus C−D bonds under the assumption that, once bound to the active site, ethane is free to rotate and present either of its carbon atoms to the active hydroxylating species. Reaction at the tritium atom will not be revealed in the ^{3}H NMR spectrum but is expected to be much slower. These experiments were carried out under V_{max} conditions, in which a saturating amount of substrate was added to the enzyme, conditions which could lead to an artificially low estimate of intermolecular isotope effects.

The ^{3}H NMR spectrum of the alcohol products formed by hydroxylation of (*S*)-[1-^{2}H$_1$,1-^{3}H]butane with sMMO is given in Figure 4A. The downfield singlet again corresponds to tritiated water. The peaks at $\delta = 3.6$ ppm arise from hydroxylation at the labeled carbon atom. The far upfield peaks correspond to hydroxylation at C2, C3, and C4. Oxidation of (*S*)-[2-^{3}H]butane by H + B/R mix leads to the product spectrum in Figure 5A. Tritiated water appears far downfield. The signal at $\delta = 3.8$ ppm arises from hydroxylation at the labeled carbon ($CH_3CT(OH)CH_2CH_3$). The inset in Figure 5A shows the remaining alcohol products. The resonance at $\delta = 1.54$ ppm arises from hydroxylation at the primary carbon next to the tritium label ($CH_2(OH)CHTCH_2CH_3$). The two signals at $\delta =$ 1.47 ppm correspond to the diastereomeric products formed from hydroxylation at the unlabeled secondary carbon. The farthest upfield resonance is due to hydroxylation of the primary carbon more distant from the tritium label.

Analysis of Derivatives. Conversion of the ethanol products to their ethyl mandelates[54] can afford five ^{3}H NMR resonances, two sets arising from diastereomers and one from another product. These signals are shown in Figure 3B. The products arising from stereochemical inversion of (*S*)-ethane are labeled 2m and 4m, those with retention are labeled 1m and 3m. The peaks correspond to mandelates of the alcohols with the same number of designations in Figure 2. The loss of stereochemistry was calculated by adding the areas of peaks 2m and 4m and dividing by the combined areas of all four peaks. The value determined was corrected for the %ee of the substrate, 88 ± 2% for ethanes, 95% for primary labeled butanes, and 99% for butanes labeled at the 2° carbon.[52] The corrected percent inversion is given by eq 4, where x is the true fraction which has undergone inversion, e is the percent of the major substrate enantiomer, f is the observed percent of products displaying retention, and ee is the percent enantiomeric excess of the substrate (e minus the percent of the minor substrate enantiomer).

$$x = (e - f)/\text{ee} \tag{4}$$

The ^{3}H NMR spectrum of the mandelate derivatives of the hydroxylation products of (*S*)-[1-^{2}H$_1$,1-^{3}H]butane are given in Figure 4B (whole spectrum) and inset (expanded version). The mandelates of the diastereomeric esters have ^{3}H NMR signals at $\delta = 3.8-3.9$ which were analyzed analogously to the ethane products above. The ^{3}H spectrum of the mandelate derivatives of alcohols formed with (*S*)-[2-^{3}H]butane is given in Figure 5B. The signal at $\delta = 4.82$ ppm comes from the mandelate of (*R*)-2-butanol, which indicates retention of stereochemistry. The $\delta = 4.86$ ppm peak is indicative of inversion to form (*S*)-2-butanol. Table 1 summarizes the results for ethane hydroxylations, Table 2 reports results for oxidations of (R) and (*S*)-[1-^{2}H$_1$,1-^{3}H]-butane, and Table 3 lists the findings for butane substrates labeled at the C2 position.

Oxidation by Purified Components. Hydroxylations of chiral ethane took place with an intramolecular k_H/k_D ratio of 3.4 ± 0.4 and a C2/C1 ratio, reflecting relative hydroxylation rates at the unlabeled versus the labeled carbon atom, of 3.3:1. The ethanol products displayed 72% retention of stereochemistry. For butanes labeled at the primary position, smaller intramolecular isotope effects were observed, together with a slightly higher ratio of hydroxylation at the unlabeled primary carbon to the one bearing the tritium atom. The amount of retention of stereochemistry was slightly larger for these alcohols than for the ethanes. The differences between results from oxidations of the (*R*)- and (*S*)-enantiomers of each substrate were within experimental error.

Oxidation by Hydroxylase and B/R Mix. When H and a mixture of B and R which contained other cellular proteins were used to hydroxylate the chiral alkanes, very low or even inverse (<1) intramolecular kinetic isotope effects were observed. This phenomenon was determined to occur not because the protein preferentially oxidized the C−D over the C−H bond but because the product distributions were disturbed by a second enzymatic process which was acting on, and even interconverting, the product alcohols. Several factors were diagnostic of this secondary process. In multiple trials, the results were more variable than when purified proteins were used. A sign that the product distributions from H + B/R mix suffered from a process that masked their true values was that the amount of

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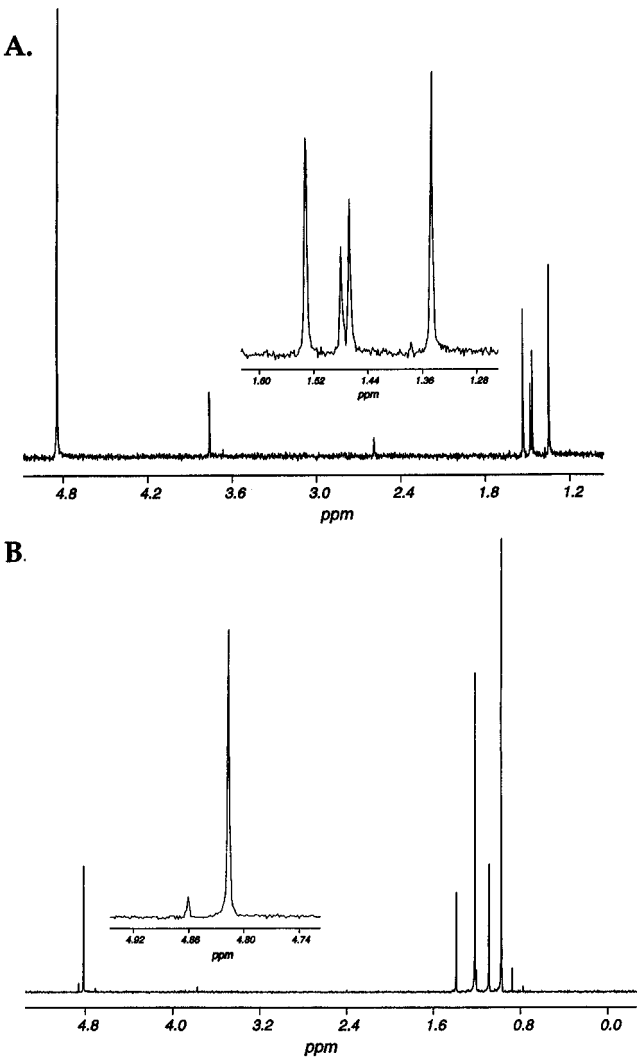

Figure 5. ^{3}H NMR analyses of the oxidation products of (*S*)-[2-^{3}H]-butane. (A) Spectrum of the alcohol products. Tritiated water appears far downfield. The product of hydroxylation at the labeled carbon appears at $\delta = 3.8$ ppm. The inset is an expansion of the upfield region, as described in the text. (B) The spectra of corresponding mandelate derivatives. The inset is an expansion of the region of the esters of the products arising from oxidation at the labeled carbon. The peak at $\delta =$ 4.86 ppm corresponds to the mandelate of (*S*)-2-butanol, signifying inversion of stereochemistry of the starting alkane. The signal at $\delta =$ 4.82 ppm arises from the (*R*)-2-butanol which results from retention of stereochemistry.

Table 1. Results of Oxidizing (*R*)- and (*S*)-[1-^{2}H$_1$,1-^{3}H]Ethane with Purified H, B, and R

substrate	(*R*)-ethane	(*S*)-ethane
k_H/k_D[a]	3.8	3.0
C2/C1[a]	3.3	3.4
% retention	72	71

[a] Calculated from the product alcohol ^{3}H NMR spectra.

Table 2. Results of Oxidizing (*R*)- and (*S*)-[1-^{2}H$_1$,1-^{3}H]Butane with Purified H, B, and R

substrate	(*R*)-butane	(*S*)-butane
k_H/k_D[a]	2.5	1.8
C4/C1[a]	3.8	3.3
% retention	76	78

[a] Calculated from the product alcohol ^{3}H NMR spectra.

retention of stereochemistry in products arising from oxidation of the C−H bond was not the same as the amount of retention in products from oxidation of the C−D bond. Details are available as Supporting Information.

Table 3. Results of Oxidizing (*R*)-, (*S*)-, and Racemic [2-^{3}H]Butane with a Mixture of H, B, and R Containing Other Cellular Proteins

substrate	(*R*)-[2-^{3}H]butane	(*S*)-[2-^{3}H]butane	racemate
% (*S*) alcohol	86.5 (retention)	4.3 (inversion)	42.8
% (*R*) alcohol	13.5 (inversion)	95.7 (retention)	57.2
C3/C2[a]	2.6	2.2	2.3
C1/C2[a]	5.4	4.6	4.8

[a] Calculated from the product alcohol ^{3}H NMR spectrum.

Retention of stereochemistry was primarily seen with both (*R*)-[2-^{3}H]butane and (*S*)-[2-^{3}H]butane (Table 3). Products obtained from a racemic mixture of (*R*)-[2-^{3}H]butane and (*S*)-[2-^{3}H]butane showed only a slight preference for hydroxylation of one enantiomer over the other (Table 3). The product ratios were 1:1.3.

Oxidations of C_2D_6 and Exchange Reactions. In order for an exchange reaction of hydrogen atoms at the α carbon atom to explain the anomalous results obtained with H + B/R mix, the process would have to be stereoselective and reversible, properties characteristic of an enzymatic reaction. The possibility that such an exchange could occur was tested by oxidizing C_2D_6 both with H + B/R mix and with purified component proteins. When the latter were used, less than 2% of the ethanol was detected by GC/MS to be CD_3CDHOH, the rest being CD_3CD_2OH. Oxidation with H + B/R mix resulted in ∼12−13% CD_3CDHOH in the product mixture. The exchange reaction is thus independent of the hydroxylation reaction. Incubation of CD_3CD_2OH in a solution containing cellular extract, NAD^+, and NADH resulted in as much as 25% exchange of deuterons for protons at the α carbon. Much less exchange (∼5%) was observed when NAD^+ was not added, and the presence or absence of hydroxylase did not affect the amount of exchange.

Discussion

The present results indicate that hydroxylation of both chiral ethane and butane substrates by sMMO from *M. capsulatus* (Bath) affords products which retain their stereochemistry to a large degree (71−96%). These data, together with other results in the literature, can be explained by a simple unifying mechanism for the hydroxylation step, as described in the following analysis.

Differences between H + B/R Mix and Purified Protein Components. A comparison of the results summarized in Tables 1, 2, S1, and S2 clearly indicates the disparity between oxidations performed using purified sMMO component proteins versus partially purified cellular extracts. Control oxidations of C_2D_6 by these two systems as well as the C−H/C−D exchange reactions observed with C_2D_5OH support the hypothesis that a cellular protein other than sMMO hydroxylase catalyzes exchange of the hydrogen atoms at the α carbon of the product alcohol. One likely such enzyme is an alcohol dehydrogenase (ADH), which catalyzes the reaction given in eq 5. Further discussion of this process is supplied as Supporting Information.

$$NAD^+ + RCH_2OH \rightleftharpoons H^+ + NADH + RCHO \quad (5)$$

Alkanes Are Not Highly Constricted in the Active Site Prior to Hydroxylation. The reaction products obtained with racemic [2-^{3}H]butane indicate clearly that the active site does not rigidly constrain the substrate, since there was only a slight discrimination between the two enantiomers of this probe. The exchange reaction described above would not affect secondary alcohols. An extremely crowded active site would most likely

readily distinguish between an ethyl and a methyl substituent at the reacting carbon atom.

Kinetic Isotope Effects. When chiral alkanes were hydroxylated by the sMMO system reconstituted from the purified components, intramolecular isotope effects in the range of 3−4 were determined, values similar to those obtained with other substrates.[43] Intramolecular kinetic isotope effects of ≈4 with these chiral substrates are similarly observed with sMMO from *M. trichosporium* OB3b.[46] In the radical clock experiments with *M. capsulatus* (Bath), an intramolecular isotope effect of ≈5 was interpreted as indicating that substantial C−H bond breakage is involved in the hydroxylation step.[43] With cytochrome P-450 hydroxylations, k_H/k_D values of 7−10 have been reported.[32] Such an isotope effect is expected to be ≥1, the precise value depending on the linearity of the O···H-C unit in the transition state[56] and on whether the transition state is early or late.[57]

The C2:C1 hydroxylation ratio depends upon the magnitude of the intramolecular kinetic isotope effect at C1, referred to as k_H/k_D, assuming that the hydroxylating species has equal access to hydrogen atoms on both the C1 and C2 positions of chiral ethane. In other words, ethane rotation would occur in the active site, rendering both ends of the molecule accessible to the active hydroxylating species. The ratio of C2/C1 products would thus reflect the relative ability to hydroxylate C−H versus C−D bonds, statistically corrected for the numbers of such bonds. From the observed k_H/k_D ratios for the chiral ethanes, 3.8 and 3.0 (Table 1), we compute a C2:C1 ratios of 2.38 and 2.25, respectively, smaller than the observed values of 3.3 and 3.4. This difference might reflect secondary kinetic isotope effects or some other factor not yet identified.

Calculation of an Apparent Rebound Rate Constant. The use of many calibrated radical probes has led to several varying values for the rate of the consensus oxygen rebound mechanism in C−H bond functionalization processes. For chiral ethane probes, the known barrier to rotation for an ethyl radical can be used to calibrate the probe at different temperatures. These data can be applied to the ratio of retention and inversion to yield an apparent rebound rate constant. No racemization occurs in the derivatization of the alcohols when the reaction takes place below −10 °C.[55]

Equation 2 yields a calculated value of $k_{rotation} = 1 \times 10^{13}$ s^{-1} at 45 °C for the ethyl radical. Therefore, when we include the data for our % retention/% inversion in eq 3, we calculate that any radical capture process must occur at approximately $k_{rebound} = 1 \times 10^{13}$ s^{-1}. This value indicates that the process is occurring on a time scale approaching that of a single bond vibration. Such an interpretation appears impossible, however, if it corresponds to the lifetime of a discrete intermediate species. We can calculate the maximum rate for any process of this type, in which $\Delta G^{\ddagger} = 0$, i.e., the decomposition of a transition state, to be 6.3×10^{12} s^{-1} at 318 K. This result leads us to conclude either that our approach to calculating the rebound rate is not a good approximation as believed or that the present consensus oxygen rebound mechanism for C−H bond functionalization is insufficient to account for hydroxylation of ethane by sMMO.

Comparisons with Literature. Very similar stereochemical results were obtained when chiral alkane was converted to product alcohols with sMMO from *M. trichosporium* OB3b.[46] The products consistently had ≈2:1 retention versus inversion of stereochemistry observed for both substrate enantiomers and for both ethane and butane substrates.[46,58] With sMMO from *M. capsulatus* (Bath), greater retention of configuration was observed and at a higher temperature, where the putative ethyl radical would rotate even more rapidly. The hydroxylase proteins from the two organisms are very similar in amino acid sequence and structure, the main differences between the two systems apparently being the rates of formation and decay of intermediates in the reaction cycle, *M. trichosporium* OB3b being the faster of the two.[15,31] Our work with the radical clock substrate probes implied a putative rebound rate constant $>10^{13}$ s^{-1} for sMMO from *M. capsulatus* (Bath) and $3-6 \times 10^{12}$ s^{-1} for sMMO from *M. trichosporium* OB3b,[43] and the results have been interpreted in terms of a nonsynchronous radical-like mechanism,[44] which is further elaborated below. Because some of the experiments with the *M. trichosporium* OB3b enzyme were carried out with identical batches of substrates reported here, it is unlikely that the different results are due to variations in the substrates or analytical procedures. When the copper-containing particulate MMO was studied by using the same methodology, hydroxylation was found to proceed with complete retention of configuration at the chiral atom.[59]

An extremely large intermolecular isotope effect (50−100) has recently been reported for the decay of the oxidizing intermediate Q in the presence of CD_4 versus CH_4 for hydroxylase from *M. trichosporium* OB3b.[30] This very large isotope effect was accompanied by intermolecular isotope effects in product distributions of ∼20 and intramolecular isotope effects of 4−13. The results were interpreted as signifying that, following hydrogen atom abstraction as depicted in Figure 1, the methyl radical reabstracts the protium or deuterium from the iron-bound hydroxyl group, regenerating a different form of the original high-valent intermediate. If the abstraction but not the radical recombination step displays a large isotope effect, this pathway might account for the discrepancy between kinetic isotope effects for reactivity of the intermediate Q toward these substrates versus the resulting product alcohols. No evidence was presented that such a complicated process exists when ethane or butane is used as substrate, and the implications of such a process on the chiral ethane results were not addressed. In the absence of a fourth isotope of hydrogen, chiral ethanes are the closest analogs to methane for which absolute stereochemistry can be determined.

Mechanistic Implications. We now inquire what, given the foregoing analysis, the experimental results would indicate if the hydroxylation step were to occur with complete retention, with racemization, or with complete inversion of stereochemistry. None of the manipulations, including the derivatization procedures, scrambles the stereochemistry.[55] In hydroxylations of chiral ethane by pMMO,[59] the products were treated by the same methodology and 100% retention of configuration was observed, providing evidence against scrambling during the workup. Total inversion of stereochemistry would suggest a concerted reaction mechanism in which the C−O bond was formed on the face opposite to the C−H bond being broken, inverting the remaining three C−X bonds (where X = C or H). Total retention would indicate that the hydroxylation proceeds in a concerted manner with formal oxygen atom insertion into a C−H bond or occurs in two successive steps: C−O or C−metal bond formation at the alkyl carbon atom followed by C−H bond cleavage or reductive elimination. Racemization at the chiral carbon atom would be indicative of a process involving a radical which had sufficient time to rotate freely before the relatively slow recombination. Branched pathways would complicate any of these interpretations.

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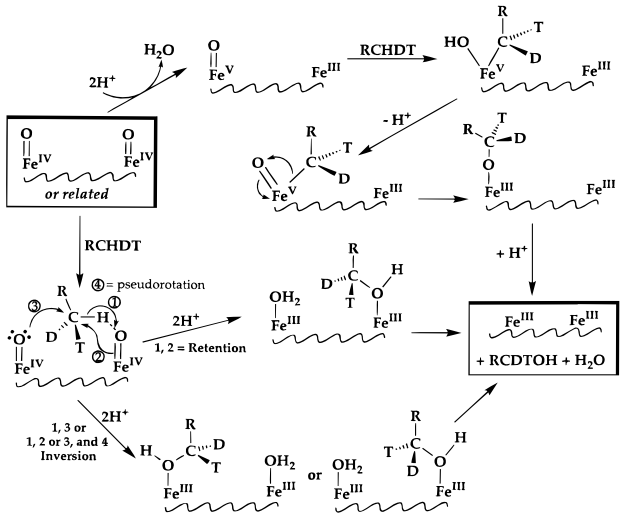

Figure 6. (Top) One alternative for the oxidation of alkane by a high valent iron-oxo intermediate without formation of a free substrate radical. This mechanism features oxygen atom insertion into an iron-bound alkane. In this scheme, protonation of one oxygen atom to form water occurs before substrate activation. It is unclear how inverted products could occur via this mechanism. (Bottom) A nonsynchronous concerted mechanism for oxidation of an alkane by sMMO. In one manifestation of this mechanism (upper arrow), the alkane molecule is doubly activated by one iron and its bound oxygen atom, leading to retention. Inversion can occur (bottom arrow) either by backside attack from the other oxygen atom (1 + 3) or by pseudorotation of a pentavalent carbon species [1 + (2 or 3) + 4]. This mechanism currently has no precedence in non-heme iron model chemistry, in which primary alkane hydroxylation by dioxygen does not occur. Instead, nonproductive decomposition pathways result.[63,64]

Given the above analysis, it is necessary to delineate a mechanism for sMMO hydroxylation of small alkanes in such a manner as to produce predominant, but not complete, retention of stereochemistry at the oxidized center. It is possible that there exists some radical or possibly carbocation character in the substrate during the hydroxylation step which inverts to a limited degree. This scenario might be interpreted in several ways. There could be parallel reaction pathways, one involving a substrate radical (as in Figure 1) and one, or more, not (as in Figure 6). The degree to which a radical versus nonradical mechanism is followed may be determined by how snugly the substrate fits into the active site. Parallel mechanistic pathways have similarly been invoked in porphyrin model systems to explain the stereochemical consequences of hydroxylation chemistry depending on steric interactions of the substrate with the catalyst.[60−62] Alternatively, abstraction of a hydrogen atom to form a substrate radical might occur in every reaction. The rate constant for the rebound reaction may be so large that only a small quantity of the substrate radical has sufficient lifetime for rotation about the C−C bond to occur before recombination with the bound hydroxyl group. Results with radical clock substrate probes were consistent with either no substrate radical formation or an extremely fast rebound step having a rate constant on the order of 10^{13} s^{-1}, too large really for formation of a discrete radical intermediate.

One proposal for the mechanism by which the oxidizing intermediate in sMMO reacts with substrate is depicted in the upper pathway of Figure 6. This scheme should result in complete retention of stereochemistry at the chiral carbon and is therefore not supported by the current results as the only mechanism at work.

Another explanation, perhaps the most satisfactory one, for the observed product distributions is that the reaction proceeds through a nonsynchronous concerted mechanism,[40,44] as presented in the lower part of Figure 6. In this representation, intermediate Q is depicted as a diiron(IV) dioxo species, although other formulations are possible. The hydrocarbon substrate is doubly activated by two iron-bound oxo groups, either one of which could evolve into the hydroxyl group of the product alcohol. In this scenario, O−H bond formation, C−H bond breaking, and C−O bond formation occur in a concerted but not totally synchronous manner. For the pathway shown on the upper arrow, preferential C−O bond formation at the oxygen atom that takes the hydrogen (1+2) would lead to predominant retention of stereochemistry at the labeled carbon. As shown on the bottom arrow, C−O bond formation at the other oxygen atom would invert the stereochemistry (1+3). Formation of a pentavalent carbon species[65] and pseudorotation could also cause inversion [bottom arrow, 1 + (2 or 3) + 4]. This mechanism is consistent with the observed kinetic isotope effects and explains the lack of ring-opened products in the radical clock studies, since a free substrate radical is never formed. It also explains the greater retention of stereochemistry for the C2 chiral butane hydroxylation; steric bulk limits backside attack and inversion of stereochemistry, and it would also retard pseudorotation. Oxidation of primary carbons preferentially over secondary over tertiary ones, the opposite trend to that expected for a radical reaction, is also explained by this mechanism, because a primary carbon atom would have the least steric restrictions to formation of such a highly ordered transition state. In the absence of direct observation of substrate intermediates, diagnostic substrates such as the tritiated chiral alkanes described here offer the most information thus far about the characteristics of the transition state.

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Supporting Information Available: A full discussion of the oxidation of [1-^{2}H$_1$,1-^{3}H]ethane and [1-^{2}H$_1$,1-^{3}H]butane by B/R mix (Tables S1 and S2) and the exchange process (Figure S1) (8 pages). See any current masthead page for ordering and Internet access instructions.

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