The Stereospecific Hydroxylation of [2,2-\textsuperscript{2}H\textsubscript{2}]Butane and Chiral Dideuteriobutanes by the Particulate Methane Monoxygenase from \textit{Methylococcus capsulatus} (Bath)*

Received for publication, January 30, 2003, and in revised form, August 8, 2003
Published, JBC Papers in Press, August 8, 2003, DOI 10.1074/jbc.M301018200

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Experiments on cryptically chiral ethanes have indicated that the particulate methane monoxygenase (pMMO) from \textit{Methylococcus capsulatus} (Bath) catalyzes the hydroxylation of ethane with total retention of configuration at the carbon center attacked. This result would seem to rule out a radical mechanism for the hydroxylation chemistry, at least as mediated by this enzyme. The interpretation of subsequent experiments on \textit{n}-propane, \textit{n}-butane, and \textit{n}-pentane has been complicated by hydroxylation at both the \textit{pro}-\textit{R} and \textit{pro}-\textit{S} secondary \textit{C–H} bonds, where the hydroxylation takes place. It has been suggested that these results merely reflect presentation of both the \textit{pro}-\textit{R} and \textit{pro}-\textit{S} \textit{C–H} bonds to the hot "oxygen atom" species generated at the active site, and that the \textit{oxo}-transfer chemistry, in fact, proceeds concomitantly with retention of configuration. In the present work, we have augmented these earlier studies with experiments on [2,2-\textsuperscript{2}H\textsubscript{2}]butane and designed \textit{d}_{1}\textit{L} form chiral dideuteriobutanes. Essentially equal amounts of [\textit{2R}][3,3-\textsuperscript{2}H\textsubscript{2}]butan-2-ol and [\textit{2R}][2,2-\textsuperscript{2}H\textsubscript{2}]butan-2-ol are produced upon hydroxylation of [2,2-\textsuperscript{2}H\textsubscript{2}]butane. The chemistry is stereospecific with full retention of configuration at the secondary carbon oxidized. In the case of the various chiral deuterated butanes, the extent of configurational inversion has been shown to be negligible for all the chiral butanes examined. Thus, the hydroxylation of butane takes place with full retention of configuration in butane as well as in the case of ethane. These results are interpreted in terms of an \textit{oxo}-transfer mechanism based on side-on singlet oxene insertion across the \textit{C–H} bond similar to that previously noted for singlet carbene insertion (Kirmse, W., and Özkir, I. S. (1992) \textit{J. Am. Chem. Soc.} 114, 7590–7591). Finally, we discuss how even the oxene insertion mechanism, with "spin crossover" in the transition state, could lead to small amounts of radical rearrangement products, if and when such products are observed. A scheme is described that unifies the two extreme mechanistic limits, namely the concerted oxene insertion and the hydrogen abstraction radical rebound mechanism within the same over-arching framework.

\footnote{This work was supported by Academia Sinica and Grants from the National Science Council of the Republic of China (NSC 89-2113-M-001-021, 89-2113-M-001-098, and 90-2113-M-001-080). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.}

\footnote{The on-line version of this article (available at http://www.jbc.org) contains Supplemental Materials.}

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\footnote{The abbreviations used are: pMMO, particulate methane monoxygenase; Pipes, 1,4-piperazinediethanesulfonic acid; GC-MS, gas chromatography-mass spectrometry; \textit{de}, diastereomeric excess; ee, enantiomeric excess.}

\footnote{This paper is available on line at http://www.jbc.org}
The Hydroxylation Chemistry of Deuterated Butanes by pMMO

EXPERIMENTAL PROCEDURES

Growth of Methanotrophs (17, 18)—M. capsulatus (Bath) (ATCC 33009) used in the studies were maintained on Petri plates containing the nitrate mineral salts medium (NMS medium) with 1.7% agar. Cultures were maintained in a closed anaerobic jar with an atmosphere of 20% methane in air and streaked onto fresh plates every 4–6 weeks. The organisms were first transferred from Petri plates to 250-ml flasks and subsequently to 2-liter Erlenmeyer flasks containing 30 and 300 ml, respectively, of the nitrate mineral salts medium with added CuSO4 (10 μM), 20% methane in air atmosphere, and continual shaking. The organism was grown for 48 h in these small scale cultures. The 300-ml cultures were used to seed a Bioflo 3000 fermentor (New Brunswick Inc.) with 5-liter fermentor vessel containing 3 liters of the above-described medium. After 24-h culturing, the optical density of the culture media was attended to 1.0–1.2 OD, based on the UV absorption at 595 nm, and the culturing volume was increased to 5 liters. A cell density up to 10 g/liter could be obtained under 1:4 methane/air ratio after several medium replenishments in which the CuSO4 concentration was increased every 24 h in 10-μM increments up to 40 μM. Methane feeding rate was controlled by the oxygen content in the culture media to around 2–5% of the dissolved oxygen at saturation.

Preparation of [2-2H1]Butan-2-ol—To a suspension of lithium aluminium deuteride (LiAlD4) (1.46 g, 34.7 mmol) in diethyl ether was slowly added butan-2-one (2.5 g, 34.7 mmol) in diethyl ether solution (5 ml) in an atmosphere of argon. The reaction mixture was stirred for 18 h at room temperature in a 250-ml round-bottomed flask. The resultant mixture was quenched by the addition of water (0.5 ml) and saturated sodium sulfate solution (3–5 ml) followed by diethyl ether (1 × 100 ml, 2 × 50 ml). After 10 min, a white precipitate was formed. The reaction mixture was then filtered through a pad of silica gel. The organic filtrate was washed with deionized water, brine, dried over anhydrous MgSO4, and concentrated to obtain the desired product (1.95 g) in 74% yield.

Preparation of [2-2H2]Butan-2-ol-p-Tosylate—To a solution of n-butyl lithium (16.3 ml, 1.6 m in hexane) in 50 ml of hexane was slowly added over a 20-min period [2-2H2]butan-2-ol (1.95 g, 26 mmol) in hexane solution in an ice bath under an atmosphere of argon. After stirring for an additional 15 min, p-tosylate chloride (4.95 g, 26 mmol) was added to the reaction mixture over a period of 10 min in an ice-water bath. The mixture was then removed and a white slurry solution was formed within 5 min. The reaction mixture was then filtered and a pad of silica gel and concentrated to obtain a colorless oil. Separation of the resulting product by column chromatography gave the tosylated butanol (2.56 g) in 60% yield.

Preparation of (2R*,3S*)-[2,3-2H3]-2,3-Butanediol Di-p-Tosylate—All three stereoisomers of 2,3-butanediol were tosylated by following the procedure reported previously with slight modification (22, 29, 30). Typically, to a solution of p-tosylate chloride in dry pyridine was added the appropriate 2,3-butanediol. A precipitate of pyridine hydrochloride appeared after 5–10 min and the resulting suspension was stirred under argon for 5.0 h at room temperature. The reaction was then quenched and worked up. (2R,3S*)-, (2R,3S*), and (2S,3S*)-2,3-butanediol di-p-tosylate butanes were obtained in yields of 78–85%. included in the analysis of the kinetic data (25, 26). The true deuterium isotope effect appears to be significantly smaller. In any case, similar experiments on cryptically chiral ethanes in the case of pMMO from M. capsulatus (Bath) have shown that the insertion of the active oxygen atom species into the C–H bond during the hydroxylation occurs with 100% retention of configuration (20). This result, together with the relatively normal kinetic isotope effect observed for hydroxylation of the C–H and C–D bonds, has provided strong argument in support of a concerted reaction pathway in the case of the membrane-bound form of the enzyme.

A third mechanism, involving the formation of a carboxylation intermediate, has also been invoked based on mechanistic probe studies on SMMO and cytochrome P450 (27). In radical clock experiments, small amounts of rearrangement products derived from a putative carbocation precursor have been observed. Such experiments are not possible in the case of pMMO, where the active site is tucked within the end of a relatively deep hydrophobic pocket and is inaccessible to the bulky mechanistic probe. Direct proton-coupled electron transfer within the ground state seems relatively unlikely for a small alkane constrained in a hydrophobic environment. This electron transfer is expected to be extremely slow because the driving force is so much larger than the reorganization energy for the process in the case of methane and related alkanes. In any case, evidence for structural rearrangements of any carboxylation intermediate should be reflected in the analysis of the stereochemical distribution of the reaction products.

The pMMO exhibits limited substrate specificity (21). Only short-chain normal alkanes (<5 carbons) are hydroxylated and similar alkenes epoxidated. Hydroxylation of n-propane, n-butane, and n-pentane favors attack at the C2 carbon. In the case of 1-butene, epoxidation across the double bond occurs to a comparable extent as hydroxylation of the C2 carbon at the other end. This regioselectivity has been accounted for in terms of the details of the presentation of the C–H bond (or the C=C bond) to the metal cluster generating the hot oxygen atom species when the alkane (or alkene) substrate is maximally inserted into the hydrophobic pocket of the active site. Obviously, either end of the hydrocarbon chain could insert into the depth of the hydrophobic pocket of the active site with essentially equal probability. The stereoselectivity observed for the hydroxylation of n-butane and n-pentane are also unusual, with a preference toward the (2R)-alcohol, and an enantiomeric excess (ee) of 46(4.5)% and 80(2)%, respectively. However, in the case of 1-butene, the bias is toward the (S)-alcohol, with an ee value of −50(4)%. These results suggest different free energies associated with the presentation of the pro-R and pro-S C–H bonds to the active site; that is, there exists a rapid equilibrium of competing substrate-binding modes. More precisely, this equilibrium corresponds to different free energies associated with binding the substrate with different orientations at the active site of the activated enzyme. Within this context, either the rate of conformational adjustment of the hydrocarbon (including rotation about its long axes) in the hydrophobic pocket of the active site, or the on-off rate of the free substrate, or both, must be fast compared with the rate of the hydroxylation chemistry.

In the present study, we have attempted to confirm these earlier findings with experiments on [2,2-2H3]butane and designed d,l form chiral dideuterated butanes. GC analysis of the stereochemical distribution of the corresponding (R)-2-acetoxy-2-phenylethanolate ester derivatives of the butanol products, together with GC-MS in conjunction with isotopic chiral labeling, have been used to follow the hydroxylation chemistry and to obtain independent and direct determinations of the extent of configurational inversion during the hydroxylation process. The results on [2,2-2H3]butane are remarkable in that essentially equal amounts of (2R)-[3,3-2H3]butan-2-ol and (2R)-[3,3-2H3]butan-2-ol are produced. The chemistry is stereospecific with full retention of configuration at the secondary carbon oxidized. In the case of the various chiral deuterated butanes, the distribution of products obtained mirrored the stereocentric configurations of the chiral butane substrates; after allowance is made for the expected deuterium isotope effect on the kinetics of the oxygen atom insertion step. The extent of inversion is shown to be negligible for all the chiral deuterated butanes examined. Thus, we conclude that the hydroxylation of butane proceeds with full retention of configuration in butane as well as in the case of ethane. These results are interpreted in terms of an αα-transfer mechanism based on side-on singlet oxene insertion across the C–H bond similar to that previously noted for singlet carbene insertion (28).
Preparation of the [2,2-2H2]Butane and Relevant Chiral [2-2H1,3-2H1]Butane—10–15 ml of 1.0 M superdeuteride (Aldrich Inc., 15 mmol, LiEt4B'H) in THF was added to a 10-ml two-necked round-bottomed flask equipped with a condenser. The solution was concentrated to ~0.5–1.0 ml in vacuo to obtain a saturated superdeuteride solution. The appropriate mono-p-tosylate butanol and di-p-tosylate butandiol (2.5 mmol) in THF solution (100 ml) was added to the reaction mixture in a closed vacuum system at 65 °C. Vigorous gas evolution occurred for 15–20 s. The reaction mixtures were refluxed by equipping the flask with a ~15 °C cooling circulating condenser and stirring for an additional 1.0 h at room temperature, and the deuterated butane gas was withdrawn from the headspace of the condenser into a 30-ml serum bottle in vacuo. In this manner, ~70% pure deuterated butanes, namely (2R)-[2,2-2H2]butane, ~15%, and normal butane, ~8%, derived from the residual hydrogen in the superdeuteride. In the case of the preparation of the [2,2-2H2]butane, the yield was only 70%, with the monodeuterated and nondeuterated butane contamination amounting to 24 and 6%, respectively.

Derivatization of the Deuterated Butan-2-ol—R)-2-acytelylmandelic acid ((R)-2-acteyloxy-2-phenylethanone) (Aldrich Inc., 47 mg, 0.24 mmol) and 4-(dimethylamino)-pyridine (DMAP) (Merck Inc., 2.0 mg) were mixed in 2.0 ml of methylene chloride at ~40 °C. The mixture was then spun down on a Sorval m film and then stirred overnight at room temperature. The suspension was filtered through a pad of silica gel. The filtrate was evaporated to ~0.30–0.38 ml under a dinitrogen stream and characterized by gas chromatography (Agilent HP6890 Plus equipped with a HP-5 capillary column, 30.0 m × 0.25 mm × 0.25 µm film thickness) to identify (b) the concerted oxo-transfer mechanism without configurational inversion at the secondary carbon(s), and (c) a rapid equilibration for presentation of the C–H or C–D bonds on the butane sample, and [(2R)-[2-2H1,3-2H1]butane] was determined by GC-MS (Finnigan G8000 series) equipped with a VG Perkin Elmer DB-1 column, ~6.0 µm × 0.25 mm × 0.25 μm film thickness) were obtained from the relevant tosylated butanol. As expected, the contaminants were monodeuterated butane (2S)- or (2R)-[2-2H2]butane, ~15%, and normal butane, ~8%, derived from the residual hydrogen in the superdeuteride. In the case of the preparation of the [2,2-2H2]butane, the yield was only 70%, with the monodeuterated and nondeuterated butane contamination amounting to 24 and 6%, respectively.

Hydroxylation of Deuterated Butanes Mediated by pMMO—To 4.0 ml of Pipes buffer (25 mM) containing 100 µM CuSO4 and 0.5 ml 3.0 mM sodium azide was added a 0.5 ml M. capsulatus (Bath) cell stock solution (grown under 30–40 µM CuSO4), and the mixture was incubated at 42 °C with a gas consisting of air and the deuterated butane at a composition of 50:50 volume ratio. After 2.0 h of incubation, the cell mixtures were withdrawn in 0.70-ml aliquots to 1.5 ml Eppendorf tubes. To the reaction mixture in each of the Eppendorf tubes, 0.70 ml of methylene chloride was added and then spun down on a Servol microcentrifuge at 8,000 rpm for 5 min. The organic layer was dried over anhydrous MgSO4 and filtered through a pad of silica gel. The filtrate was evaporated to 0.30–0.80 ml under a dinitrogen stream and characterized by gas chromatography (Agilent HP6890 Plus equipped with a HP-5 capillary column, 30.0 m × 0.25 mm × 0.25 µm film thickness) to identify the chiral deuterated butanes.

Synthesis of [2,2-2H2]Butane and Chiral Deuterated Butane—Hydroxylation of C2 in propane, n-butane, and n-pentane without inversion of configuration at the carbon center would lend strong support of the proposed concerted mechanism in the case of methane and ethane. Accordingly, in this study, we have augmented our earlier pMMO studies on n-butane and n-pentane with additional experiments on [2,2-2H2]butane and chiral deuterated butane.

Various designed deisotearomeric tosylated butandiols with specific configurations were synthesized from the (2R,3S)-, (2S,3S)- as well as meso-butadiol in 80, 85, and 78% yields, respectively. After the S2 displacement (29, 30) of the tosylate group by the deuteride from the lithium triethylborodeuteride, (2S,3S)-, and (2R,3R)-[2-2H1,3-2H1]butanes as well as (2R,3S)-, and (2S,3R)-[2-2H1,3-2H1]butane were obtained (note the inversion of configuration at each of the secondary carbon centers following the reduction to give the corresponding butane).

For the synthesis of the [2,2-2H2]butane, butane-2-one was reduced with lithium aluminum deuteride and the resulting [2,2-2H2]butan-2-ol was tosylated to obtain the tosylated butanol in 75% yield. The latter was then reduced by superdeuterides to give the [2,2-2H2]butane by using the same strategy as for the synthesis of the diastereomeric butanes. All the deuterated butanes showed only two groups of signals in the 1H NMR spectrum at 0.90 and 1.30 ppm, which were assigned to the methyl and methylene groups, respectively. The most abundant molecular mass peak from the butanes synthesized appeared at m/z 60, which was readily identified as...
The equilibrium constants ($K_{\text{SS}}$ and $K_{\text{RS}}$) and kinetic isotope effects ($k_{\text{H}}/k_{\text{D}}$) derived from analysis of the distribution of (S)- and (R)-butan-2-ol esters of (R)-O-acetylmandelic acid (S/R) formed from the (S)- and (R)-butan-2-ols during hydroxylation of the various deuterated butanes. The data have been corrected for contributions from the background mono-deuterated and non-deuterated butanes.

The dideuterated butane. Monodeuterated butanes were identified at m/z 59, and nondeuterated butane or n-butane was recorded at m/z 58 as well. The efficiency of the deuteration to yield the desired dideuterated butanes was apparently only 70–80%. Since the superdeuteride used in the reduction of the ditosylates was 95–98% enriched in $^2$H, the rate of hydrogenation must be significantly faster than the deuteration process. In any case, the presence of the monodeuterated and nondeuterated background in the dideuterated substrates did not materially compromise the subsequent analysis of the data on the stereochemical distribution of the butan-2-ol esters, as the isotopomeric compositions of the various deuterated butane substrate samples were known from mass spectrometry.

**Activity Assays of $[2,2-2^H_2]$Butane and the Chiral Deuterated Butanes**

The synthesized $[2,2-2^H_2]$butane and the chiral deuterated butanes were mixed with equal volume of air and used as substrates for pMMO isolated from *M. capsulatus* (Bath) cells. These cells were cultured in NMS buffer with high copper content (30–40 μM CuSO₄). After 2 h of incubation at 42 °C in the presence of 3.0 mM sodium formate, the oxidation products, namely, deuterated-Butane 2-ols, were extracted and identified. The products revealed only hydroxylation at either the C2 or C3 position of the butanes. To evaluate the stereoelectivity of the C–H activation at the C2 or C3 carbon, the stereochemistry of the relevant oxidation products was determined by GC analysis of the corresponding (R)-O-acetylmandelic acid butan-2-ol ester derivatives (31). The ee or de measured for the products derived from hydroxylation of $[2,2-2^H_2]$butane, and the (2S,3S), (2R,3R), and (2R*,3S*)-[2-2H₂]butane samples at 42 °C were 90 (2%), 90 (8%), 19 (2%), and 64 (8%), respectively. These results may be compared with the corresponding ee of 70 (8%) determined for the (R)- and (S)-butan-2-ols derived from the hydroxylation of n-butane by pMMO in this study.

**Identification of the Products**—Mass spectrometry was used to verify the identity of the product butanols and their configurations. Mass ions from CH₃CH₂CD⁺⁺CH⁺⁺[CH₂CH₂CH₂] and CH₃CH₂CD⁺⁺CH⁺⁺[CD⁺⁺CH₂][CH₂CH₂CH₂] fragments at m/z 58, and CH₃CH₂[CD⁺⁺CH⁺⁺]CH⁺⁺ and CH₂CD⁺⁺CH⁺⁺CH₂ at m/z 59, were readily identified in the GC-MS analysis of the butanol esters of (R)-O-acetylmandelic acid derived from hydroxylation of the various deuterated butanes. In addition, we observed ions that could be assigned to CH₃CH₂CD⁺⁺CH⁺⁺ and CH₂CD⁺⁺CH⁺⁺CH₂ (m/z 57) due to hydroxylation of the nondeuterated butane in the samples. We also expected contributions of CH₃CH₂CD⁺⁺CH⁺⁺ and CH₃CH₂CD⁺⁺CH⁺⁺CH₂ to the hydroxylation of the background mono-deuterated and non-deuterated butanes.

**Summary of Kinetic parameters**

| Substrate                  | ee or de, % | $K$          | $K_{\text{insertion}}$ | $k_{\text{H}}/k_{\text{D}}$ |
|----------------------------|-------------|--------------|------------------------|-------------------------------|
| n-butane                   | 70 ± 8      | 0.17 ± 0.06b | 1.0                    | 5.5 ± 2.0f                   |
| (2R*,3S*)-[2-2H₁,3-2H₁]butane | 65 ± 6      | 0.21 ± 0.05f | 1.0                    | 5.5 ± 2.7f                   |
| (2S,3S*)-[2-2H₁,3-2H₁]butane | 94 ± 6      | 0.17 ± 0.06d | 1.0                    | 5.2 ± 0.3f                   |
| (2R,3R*)-[2-2H₁,3-2H₁]butane | −13 ± 3     | 0.25 ± 0.07b | 1.0                    | 5.5 ± 2.0                   |
| [2,2-2H₂]butane            | 97 ± 3      | 0.04 ± 0.02b | 2.8 ± 0.9b             | 5.5 ± 2.0                   |

a $S/R = (1 - ee)/(1 + ee)$ or $(1 - de)/(1 + de)$.

b $K_{\text{RS}}^{\text{H/De}} = (S/R)\text{butane}^{-1}$.

c $K_{\text{SS}} = (S/R)\text{butane}^{-2}$ assumed.

d $K_{\text{RS}} = (2K_{\text{SS}} - K_{\text{RS}}^{\text{H/De}})$ assumed.

$K_{\text{CH}_{3}\text{CH}_{2}^{\text{H}}/\text{De}}$

$K_{\text{CH}_{3}\text{CH}_{2}^{\text{De}/\text{H}}}$

$K_{\text{insertion}} = f_{\text{CH}_{3}\text{CD}_{2}/\text{CD}_{2}\text{CH}_{3}}$

$K/k_{\text{H}} = (S/R)^{\text{H/De}} (K_{\text{SS}})^{-1}$.

$K/k_{\text{D}} = (S/R)^{\text{De}/\text{H}} (K_{\text{RS}})^{-1}$.

Estimated determined for (2S,3S*)-[2,2-2H₂]butane, (2R*,3S*)-[2-2H₁,3-2H₁]butane, and (2R,3R*)-[2-2H₁,3-2H₁]butane (see Tables II, IV and V in Supplemental Materials for the details of the corrections, including the parameters in the model that provide the best fits to all the experimental data). The corrected ee or de ratios for (2,2-2H₂)butane, (2S,3S*)-[2-2H₁,3-2H₁]butane, and (2R*,3S*)-[2-2H₁,3-2H₁]butane are 96 (2%), 94 (6%), and 69 (13%) (Table I) compared with the apparent values of 90 (2%), 90 (8%), and 69 (9%) respectively. The correction to the apparent ee or de can be seen to be quite minor.

The correction in the case of (2R,3R*)-[2-2H₁,3-2H₁]butane is, as the observed or apparent de is 19 (2%), somewhat in favor of the (R)-butan-2-ols. The latter arises mostly from the hydroxylation of the background monodeuterated and nondeuterated butanes. The actual de of the butan-2-ols produced from (2R,3R*)-[2-2H₁,3-2H₁]butane is, in fact, negative (~13%; see Table I). That is, the hydroxylation of (2R,3R*)-[2-2H₁,3-2H₁]butane favors the production of the (S)-butan-2-ol from

**Correction of the Apparent de for Contributions from the Monodeuterated and Nondeuterated Butanes in the Dideuterated-Butane Samples**—As noted above, the ee or de ratios measured from GC are apparent values, and it is necessary to correct the products for the contributions introduced by hydroxylation of the mono- and the nondeuterated butanes in the dideuterated butane samples. Since the outcome of the present study supports the concerted mechanism with full retention of configuration at the carbon center hydroxylated and a rapid equilibrium for presentation of the C–H or C–D bonds on the Re and Si faces to the active site for oxygen atom transfer, we have used this model to make the corrections for the sake of consistency. Other models are, of course, possible, but in the final analysis, we must look to self-consistency as the criterion for the validity of a given model. In any case, at the level of these contaminant contributions, the corrections are actually quite small, of the order of only a few percent (within limits of experimental error) in the case of (2S,3S*)-[2-2H₁,3-2H₁]butane, (2R*,3S*)-[2-2H₁,3-2H₁]butane, and (2R,3R*)-[2-2H₁,3-2H₁]butane (see Tables II, IV and V in Supplemental Materials for the details of the corrections, including the parameters in the model that provide the best fits to all the experimental data). The corrected ee or de ratios for (2,2-2H₂)butane, (2S,3S*)-[2-2H₁,3-2H₁]butane, and (2R*,3S*)-[2-2H₁,3-2H₁]butane are 96 (2%), 94 (6%), and 69 (13%) (Table I) compared with the apparent values of 90 (2%), 90 (8%), and 69 (9%) respectively. The correction to the apparent ee or de can be seen to be quite minor.
this dideuterated butane. Again, the individual contributions could be estimated from the composition of the butane isotope-pomers in this dideuterated butane sample. The details of the corrections are summarized in Table III in Supplemental Materials.

Equilibrium Constants for the Competing Substrate Stereselective Binding Behavior and the Kinetic Isotope Effect—The above stereochemical findings are consistent with the competing substrate binding modes proposed earlier, namely, the substrate butane can become hydroxylated by presentations of the C–H or C–D bonds on the Re and Si faces of the molecule to the active oxygen atom species at the active site for o xo-transfer to the C–H or C–D bonds presented. Indeed, the distribution of products obtained for the various chiral butanes mirrors the stereochemical configurations of the chiral butane substrates, after allowance is made for the expected deuterium isotope effect on the kinetics of the oxygen atom insertion step.

We have analyzed the de ratios of the butan-2-ols observed for the various chiral dideuterated butanes within the context of this simple picture. As in the case of the n-butane reported earlier (21) the distribution ratio of the product (R)- and (S)-butan-2-ols observed on the GC chromatogram for the (2R*,3S*)-[2-2H1,3-2H1]butane should reflect the equilibrium associated with the presentation of the C–H and C–D bonds on the Re and Si faces to the active oxygen species. The true de of the butan-2-ol products was determined to be 65(8)% for this butane (Table I). This result would give an effective equilibrium constant (KRS) of 0.21(0.05), which is in excellent agreement with the value of 0.17(0.06) determined for KSi/Re from the ee value (70(8)%)) measured for butane (Table I).

However, since the two ends of this butane molecule are non-equivalent, in principle, two equilibrium constants are involved, one associated with presenting the CH3-C-H(DRe), center to the metal cluster of the activated enzyme at the active site, namely, KCHD, and the other, the CH3-C-D(DSi) end, namely KCDH. In fact, the effective equilibrium constant KRS ≈ 0.21(0.05) mentioned earlier is merely a simple average of KCHD and KCDH, an outcome that is readily verified by the equations in Table III in Supplemental Materials. Since KCHD ≈ KCHD (0.17), as highlighted in Fig. 1, we obtain KCHD ≈ 0.25 from these results.

Similarly, it can be shown that in the case of (2R,3R)-[2-2H1,3-2H1]butane and (2S,3S)-[2-2H1,3-2H1]butane, the de of the product (R)- and (S)-2-butanol are related to the products of the same equilibrium constant (K) and the kinetic isotope effect (kH/kD) of the hydroxylation as follows: (R/KR)-1(kH/kD)−1 in the case of (2R,3R)-[2-2H1,3-2H1]butane, and (KSS)-1(kH/kD) for (2S,3S)-[2-2H1,3-2H1]butane (see Fig. 2). From similar stereochemical arrangements and steric interactions shared between the CH3-C-H(DRe), end of n-butane, and the CH3-C-D(DSi) end of (2S,3S)-[2-2H1,3-2H1]butane when they are tucked in within the hydrophobic cavity of the active site (see Fig. 1), it is reasonable to assume that KSi/Re ≈ KSS. To a good approximation, steric interactions between the CH3-C-H(DRe), moity and the walls of the hydrophobic pocket should be similar for (2R,3R)-[2-2H1,3-2H1] butane and (2R*,3S*)-[2-2H1,3-2H1]butane (if secondary interactions between the two ends of the butane molecule could be ignored). If so, we expect KRR ≈ KCHD = (KRS − KSS). Thus, we have combined the de or ee data derived for (2R,3R)-[2-2H1,3-2H1]butane and (2R*,3S*)-[2-2H1,3-2H1] butane, and for (2S,3S)-[2-2H1,3-2H1] butane and n-butane, to derive the kinetic isotope effect kH/kD. In this manner, kH/kD of 5.5 ± 2.7 and 5.2 ± 0.3 were obtained for hydroxylation of the (2S,3S)- and (2R,3R)-[2-2H1,3-2H1]butane, respectively (Table I). The kinetic isotope effect kH/kD is not well determined for the (2S,3S)-[2-2H1,3-2H1]butane, as there is only limited hydroxylation of the C–D bond on the Si face here because of the kinetic isotope effect and their relatively improbable exposure to the hot oxygen species at the active site. Nevertheless, given the diversity of systems, the agreement among the different butanes is quite remarkable, lending credence to the picture depicted here.

The results obtained for [2,2-2H2]butane offer an interesting, if not surprising, contrast (Fig. 3). Here the average de ratio of 97(3)% for this butane indicates that essentially only the (2R)- butan-2-ol is formed. The same conclusion is reached whether the hydroxylation of the CH3CH2− or the CH3CD2− ends of the butane molecule is considered. As the two ends of the molecule are now distinct, we could consider the hydroxylation of CH3CH2− and the CH3CD2− separately. From analysis of the stereochemical data (GCMS data at m/z 58 and 59) within the same framework as that for the other butanes, an upper limit of ~0.03 could be derived for KCHD zab from the data for m/z 59; the data fitted best with KCHD zab ≈ 0.01 for the data of m/z 58. Here, KCHD zab denotes the equilibrium constants for presenting the C–H and C–D bonds on the Si and Re faces of the hydrocarbon to the active site. Thus, hydroxylation of the molecule yields essentially the R-alcohol only.

As noted above, it is not possible to determine the kinetic isotope effect kH/kD from the stereochemical data without invoking some assumptions about the K values. However, in the case of [2,2-2H2]butane and (2R*,3S*)-[2-2H1,3-2H1] butane, kH/kD could be inferred directly from the production rates of the product butan-2-ols determined from the GCMS data at m/z 58 and 59, after allowance is made for contributions from the 2-butanol of the same mass derived from the mono- as well as the nondeuterated alkanes and the appropriate 13C iso-
In this manner, we have obtained $k_H/k_D = 5.5(2)$ and $k_H/k_D = 2.0(0.2)$ for hydroxylation of $\text{(2R,3S)}-[2-2^\text{H}_1,3-2^\text{H}_1]\text{butane}$ and $\text{(2R,3R)}-[2-2^\text{H}_1,3-2^\text{H}_1]\text{butane}$, to the active oxygen atom species for concerted oxene transfer.

**FIG. 2.** Presentation of the Re face and Si face of the C-H and C-D bonds in $\text{(2S,3S)}-[2-2^\text{H}_1,3-2^\text{H}_1]\text{butane}$ and $\text{(2R,3R)}-[2-2^\text{H}_1,3-2^\text{H}_1]\text{butane}$, to the active oxygen atom species for concerted oxene transfer.

![Diagram of the Re face and Si face of the C-H and C-D bonds in butane](image)

These expressions allow us to relate the apparent $k_H/k_D$ (or $k_H/k_D$) deduced from the analysis of the products contributing to $m/z$ 58 in the GCMS that originate from the $[2,2-2^\text{H}_2]\text{butane}$ and the background monodeuterated butane, we obtain $k_D = 4.0k_D$ (1), $k_H = 8.0k_D$ (2), $K_{\text{insertion}} = f_{\text{CH3CD2}}/f_{\text{CH3CH2}} = 1.9 (0.9)$. $K_{\text{insertion}} = f_{\text{CH3CD2}}/f_{\text{CH3CH2}} = 1.9 (0.9)$. The process is 98–99% chiral selective, almost totally biased toward the $R$-stereoisomer, with $K_{\text{CH3CH2}} = 0.003$ and $K_{\text{CH3CD2}} = 0.01$. Other parameters required to best fit the product throughputs and their diastereomeric distributions observed in the GC and GCMS: ($K_m/K_m = 5.4$; and $k_H/k_D = 5.5$, $k_D$ denotes the $k_H$ for hydroxylation of the CD bond in the background monodeuterated butane in the sample.

**FIG. 3.** Hydroxylation of $[2,2-2^\text{H}_2]\text{butane}$, including the equilibrium for presentation of the CH$_3$CD$_2$– and CH$_3$CH$_2$– ends of the butane substrate to the active oxygen atom species in the hydrophobic pocket of the enzyme. $K_{\text{insertion}} = f_{\text{CH3CD2}}/f_{\text{CH3CH2}} = 2.8(0.9)$. The process is 98–99% chiral selective, almost totally biased toward the $R$-stereoisomer, with $K_{\text{CH3CH2}} = 0.003$ and $K_{\text{CH3CD2}} = 0.01$. Other parameters required to best fit the product throughputs and their diastereomeric distributions observed in the GC and GCMS: ($K_m/K_m = 5.4$; and $k_H/k_D = 5.5$, $k_D$ denotes the $k_H$ for hydroxylation of the CD bond in the background monodeuterated butane in the sample.

From the analysis of the products contributing to $m/z$ 58 in the GCMS that originate from the $[2,2-2^\text{H}_2]\text{butane}$ and the background monodeuterated butane, we obtain $k_D = 4.0k_D$, which when combined with the apparent isotope effect $k_H/k_D$, above, gives also $k_H = 8.0k_D$. In essence, we have compared the throughput levels of $[3,3-2^\text{H}_2]\text{butan-2-ol}$ and $[2-2^\text{H}_1]\text{butan-2-ol}$ estimated from the intensities of the mass peaks at $m/z$ 58 and 58 to relate $k_H$ and $k_D$`, and have used the level of oxidation of the background monodeuterated butane as an internal reference to quantify the production of the $[2-2^\text{H}_1]\text{butan-2-ol}$ from $[2,2-2^\text{H}_2]\text{butane}$. These expressions allow us to relate the ap-
parent $k_{\text{H}}$ and $k_{\text{D}}$ for the deuterated butane to its intrinsic $k_{\text{out}}$ for hydroxylation of the C–H and C–D bonds ($k_{\text{H}}$ and $k_{\text{D}}$), its Michaelis-Menten constant ($K_m$), and the fractional exposures of the $\text{CH}_3\text{CD}_2^-$ and $\text{CH}_3\text{CH}_2^-$ ends of the molecule ($f_{\text{CH}_3\text{CD}_2}$ and $f_{\text{CH}_3\text{CH}_2}$) to the corresponding $k_{\text{f}}$ and $K_m$ for the monodeuterated butane. Thus, we have Equations 1 and 2.

$$h'_{\text{f}} = k_{\text{f}} (K_m/K_{m'}) f_{\text{CH}_3\text{CD}_2} = 8.0 k_p$$  \hspace{1cm} (Eq. 1)

$$h'_{\text{f}} = k_{\text{f}} (K_m/K_{m'}) f_{\text{CH}_3\text{CH}_2} = 4.0 k_p$$  \hspace{1cm} (Eq. 2)

From these equations, it is apparent that any differences in the Michaelis-Menten constant ($K_m$) between the deuterated and monodeuterated butane would drop out of the apparent kinetic isotope effect $k_{\text{H}}/k_{\text{D}}'$, and we would obtain $k_{\text{H}}/k_{\text{D}}' = (k_{\text{H}}/k_{\text{D}}') (f_{\text{CH}_3\text{CD}_2}/f_{\text{CH}_3\text{CH}_2}) = 2.0$, or $(k_{\text{H}}/k_{\text{D}}') K_{\text{inversion}} = 2.0$. In the absence of any secondary isotope effects, $k_{\text{H}}/k_{\text{D}}'$ should be similar to the kinetic isotope effect observed for the other deuterated butanes, of the order of 5.5. If this is the case, we deduce a value of 2.8(0.9) for $K_{\text{inversion}}$. We could check for self-consistency by returning to Equations 1 and 2 above. From Equation 2, $K_{\text{inversion}} = 2.8$ would predict a value of 5.4 for $(K_m/K_{m'})$. From Equation 1, we obtain a value of 5.5 for the ratio of the Michaelis-Menten constants, if $k_{\text{H}}/k_{\text{D}}'$ is set to $k_{\text{H}}/k_{\text{D}}' = 5.5$. Our data are thus consistent with an intrinsic kinetic isotope effect $k_{\text{H}}/k_{\text{D}}'$ of $5.5(2.0)$ for [2,2-2H][butane].

**Overall Production Rates of the Product Butan-2-0ls for the Various Deuterated Butane Samples**—The overall rate of hydroxylation of a butane species varies with the number of deuterium atoms in the substrate because of the $k_{\text{H}}/k_{\text{D}}'$ kinetic isotope effect. Typically, there is a kinetic enrichment of the products derived from hydroxylation of the butanes with the greater number of hydrogens. In addition, these throughput rates depend on the location of the deuterium atom(s) in the chiral butane due to the different presentation of the C–H and C–D bonds on the Re and Si faces to the active oxygen atom at the active site, as well as the different insertion of the $\text{CH}_3\text{CH}_2^-$ and $\text{CH}_3\text{CD}_2^-$ ends of the butane molecule as in the case of [2,2-2H][butane]. Thus, the fractional product throughput from the deuterated butanes are 0.75 and 0.60 for the (2S,3S)-[2-2H,3-2H][butane] and (2S,3R)-[2-2H,3-2H][butane] samples, respectively. In these samples, the 2-butanol products derived from monodeuterated butane exceeded the composition of 8% in the original butane sample, whereas those derived from the deuterated butane fall below its original composition of 77% in the sample. However, this kinetic enrichment of the products derived from the lighter butanes is significant only for the (2R,3R)-[2-2H,3-2H][butane] sample, where the butan-2-ol product throughput from the deuterated butane is significantly slower. In the case of the (2S,3S)-[2-2H,3-2H][butane], the enrichment amounts to no more than 1–2%. Similar considerations pertain to (2R,3S)-[2-2H,3-2H][butane].

On the other hand, the reverse is obtained in the case of [2,2-2H][butane]. Here, we have observed a significant enrichment of the products derived from the hydroxylation of the deuterated butane instead. The fractional product throughput from the deuterated butane was 0.76, far exceeding the composition of 70% in the butane sample. This remarkable turnover in the behavior of the [2,2-2H][butane] sample relative to the other butane samples arises from the almost 6-fold decrease in the $K_m$ for [2,2-2H][butane] relative to the monodeuterated butane ($K_m$ has been assumed to be the same for all the di-, mono-, and nondeuterated butanes up to this point), as well as the almost 3-fold preferential insertion of the $\text{CH}_3\text{CD}_2^-$ end of the molecule into the active site. Evidently, [2,2-2H][butane] is a far better substrate compared with the other butanes for pMMO, presumably because of its somewhat smaller overall size and the asymmetry in its cross-section between the two ends (narrower at the $\text{CH}_3\text{CD}_2^-$ end).

As noted earlier, from the overall production levels of [3,3-2H][butan-2-ol] and [2-2H][butan-2-ol] estimated from the throughputs of the corresponding ester products at $m/z$ 59 and 58, we obtain $k_{\text{H}}/k_{\text{D}}'$ of 2.0(0.2), and from the product throughputs of [2-2H][butan-2-ol] and [3-2H][butan-2-ol] from hydroxylation of the [2,2-2H][butane] and the monodeuterated [2-2H][butane], respectively, we obtain $k_{\text{H}}' = 4.0 k_p$. Combining these results, we obtain $k_{\text{H}}' = 8.0 k_p$. Thus, although hydroxylation of the C–D bond must be intrinsically slower than the C–H bond, the smaller $K_m$ and the preferential insertion of the $\text{CH}_3\text{CD}_2^-$ end of the molecule relative to the $\text{CH}_3\text{CH}_2^-$ end has led to a significantly higher molecular throughput for [2,2-2H][butane] compared with the mono- and nondeuterated butane background; 40% faster than the monodeuterated butane and 15% faster than normal butane.

**Analysis of the Diastereomeric Distribution of the Chiral Butanol Ester Products**—The products of the pMMO-mediated hydroxylation of the (2S,3S)-[2-2H,3-2H][butane], (2R,3R)-[2-2H,3-2H][butane], and [2,2-2H][butane] samples are summarized in Table VI in Supplemental Materials. Both GC and GC-MS have been used to analyze the esters derived from the product butan-2-ols produced by the hydroxylation. Since the GC peaks are mass analyzed in the GC-MS, the stereochemical configuration of the fragment ions could be identified at each mass. As expected, when the R- and S-stereoisomers are dispersed according to their molecular masses, the data are more sensitive to details of the hydroxylation chemistry. Accordingly, we have highlighted the diastereomeric distribution of the chiral butanol ester products according to the butane precursor as well as the molecular mass to allow the reader to gauge the relative contributions of the various species to the GC as well as the GC-MS. These results are taken from the analyses summarized in Tables II, III, and V in Supplemental Materials. Only the products derived from hydroxylation of (2S,3S)-[2-2H,3-2H][butane], (2R,3R)-[2-2H,3-2H][butane], and [2,2-2H][butane] samples are analyzed in detail by GC-MS.

As summarized in Table VI in Supplemental Materials, it is possible to account for the experimental diastereomeric distribution of the chiral butanol ester products for (2S,3S)-[2-2H,3-2H][butane], and (2R,3R)-[2-2H,3-2H][butane] and [2,2-2H][butane] samples within the simple Michaelis-Menten model that we have developed to analyze the products, without invoking any configurational inversion at the secondary carbon oxidized for all the butanes. We are able to best fit all the diastereomeric data, including the overall de or ee for the sample and the diastereomeric distribution at both $m/z$ 59 and 58, assuming the same $K_m$ for the deuterated butanes as well as the background monodeuterated and nondeuterated butane, $K^{\text{H/Re}} = 0.20$ for both secondary carbons of the molecule, and a kinetic isotope effect ($k_{\text{H}}/k_{\text{D}}'$) of 5.5.

In order to rationalize the experimental data obtained for the [2,2-2H][butane] sample, it is only necessary to amend the simple Michaelis-Menten model by allowing for a different $K_m$ for this deuteriobiolute relative to the other deuterated butanes considered in this study, and including the possibility of different probability for the insertion of the two ends of the molecule into the hydrophobic pocket. Apparently, [2,2-2H][butane] is a far better substrate for pMMO compared with other butanes, but this substrate is so constrained within the active site that for the most part the C–H-Re or C–D-Re is hydroxylated ($K^{\text{H/Re}} \approx 0.01–0.03$).

(2S,3S)-[2-2H,3-2H][Butane]—In the case of (2S,2S)-[2-2H,3-2H][butane], analysis of the butan-2-ol ester products that contribute to the GC predicts an overall de of 90%,
which is in excellent agreement with the observed overall de of 90(8) % (Fig. 4a and Table II).

In the GC-MS, the (2S,3S)-[2-2H1,3-2H1]butane sample reveals primarily the product expected from the (R)-butan-2-ol formed by hydroxylation of (2S,3S)-[2-2H1,3-2H1]butane at the C–H Re, as evidenced by an obvious mass peak only at m/z 59, corresponding to the mass ion (CH3C2H10D-CH(D)CH3) (Fig. 4b). However, both the R- and S-stereoisomers show a mass signal at m/z 59 in the GC-MS chromatogram. The mass peak for the R-stereoisomer arises predominantly from (2R,3S)-[2-2H1,3-2H1]butan-2-ol ester (70% of the total products, after allowance is made for the 13C isotopomer). In addition, there is a small contribution from the 13C-isotopomers from the butan-2-ols produced by hydroxylation of the background monodeuterated butane at the Re face (0.64%). However, the S-stereoisomer at m/z 59 could only be assigned to ester products derived from: (i) the 13C-isotopomer of (2S,3S)-[2-2H1,3-2H1]butan-2-ol formed by hydroxylation at the C–D Si of the dideuterated substrate.
The GC-MS at dominantly to the mass peaks at quite good agreement with experiment. 1.6(0.3)% for the inversion during hydroxylation of C–H peak at tetrad butane in the sample would contribute mass ion (CH3C-Si58. All total, the monodeuterated butane and the nondeuterated butanes are contributing almost as much to here is actually quite complicated since the monodeuterated and nondeuterated butanes are contributing almost as much to the overall rate of production of 2-butanol as the deuterated butane despite the latter’s relatively higher abundance (77%) in the sample (See Table VI in Supplemental Materials for details). In any case, the analysis summarized in Table III in the Supplemental Material predicts an overall de of 16%, in quite good agreement with experiment. In the GC-MS, both the 2R- and 2S-stereoisomers contribute dominantly to the mass peaks at m/z 58 and 59, respectively. The GC-MS at m/z 58 corresponds to the mass ion (CH3C-H-CH(D)CH3). The R peak is dominated by two contributions: (i) the (R)-butan-2-ol ester product formed by hydroxylation of (2R,3R)-[2-2H1,3-2H1]-butane at C-D58 (25% of the total products after subtracting the contribution from the 13C isoto- pomer), and (ii) (2R)-[2-2H1]-butanol derived from hydroxylation of the prochiral CH2 on the Re side of the monodeuterated butane (13% after subtracting the 13C isoto- pomer). There is also a small contribution (0.66%) from 13C isoto- pomer of the (2R)-butan-2-ol formed from hydroxylation of C-D58 at the prochiral CH2 center of both the monodeuterated and nondeuterated butanes. Thus, together, 39% of the products contribute to the R peak at m/z 58. Hydroxylation of the monodeuterated butane at the chiral C-H(D)58(De) end on the Si face would generate the (2S)-butan-2-ol contributing 3.2% to the S peak, as would products arising from hydroxylation of the prochiral CH2 on the Si face (2.3%). Similarly, hydroxylation of the nondeuterated butane at the Si face contributes 0.11% to the S peak at m/z 58. All total, the monodeuterated butane and the nondeut- erated butane in the sample would contribute 5.5% to the S peak at m/z 58. This analysis predicts a S/R ratio of 13% for the GC-MS peaks at m/z 58, without the consideration of any configurational inversion during hydroxylation of the C-De at C2 in (2R,3R)-[2-2H1,3-2H1]-butane. From the intensity ratio of the R and S mass fragments (Fig. 4d), we obtain a relative abundance of 11 (3%) for the (2S)-butan-2-ol ester products. Thus, within experimental error, there is no evidence for configurational inversion during hydroxylation of the C-De at C2 in (2R,3R)-[2-2H1,3-2H1]-butane.

Similarly, the GC-MS at m/z 59, which corresponds to the mass ion (CH3C-D-CH(D)CH3) is dominated by the fragment from the (2S,3R)-[2-2H1,3-2H1]-butan-2-ol ester product derived from hydroxylation of the substrate at C-H(D)59 (33% after subtracting its 13C isoto- pomer), with an accompanying minor R-configuration fragment from 13C-isotopomers (1.9%) of the (2R,3R)-[3-2H1]-butan-2-ol ester product formed from hydroxylation of the C-De in the deuterated butane and hydroxylation of the C-H(D) at C3 in the monodeuterated butane. From this analysis, we predict a S/R of 6% for the relative intensity of the R and S peaks at m/z 59, which is within experimental error of the observed ratio of 11(5%) (Fig. 1e). Thus, there is no reason to invoke any configurational inversion during the hydroxylation chemistry. 

**[2,2-2H2]Butane**—The situation is relatively straightforward here. With the observed ee for the butan-2-ol products approaching 90%, primarily the R-alcohol is observed in these experiments. The (2R)-[3-2H1]-butan-2-ol is essentially the only product (49% of the total products) when [2,2-2H2]butane is hydroxylated at the CH2 center; the corresponding 25-stereo isomer accounts for a mere 1.5% of the total products. The (2R-[2-2H1]-butan-2-ol is also the principal product (35% of total products) when [2,2-2H2]butane is hydroxylated at the CD2 end, with the (2S)-stereoisomer accounting for only 0.27%. The remaining products originate from the background butanes: 18% comes from hydroxylation of monodeuterated butane, and 6.0% from hydroxylation of nondeuterated butane. (See Table VI in Supplemental Materials for details). This analysis predicts an overall de of 90% in perfect agreement with experiment.

The ester products associated with these butan-2-ols contribute to the mass peaks at m/z 59 and 58 in the GCMS, respectively. At m/z 59, the R peak arises mainly from hydroxylation of the CH3CH2- end of the butane molecule (47%); there is a small contribution from 13C isotopomers of (2R)-[3-2H1]-butan-2-ol and (2R)-[2-2H1]-butan-2-ol (1.7% total). The S peak at m/z 59 could arise only from: (i) the S-alcohol formed from hydroxylation of C-H(D) at the prochiral CH2 center of the [2,2-2H3] butane (1.4%); (ii) the 13C isotopomers of both the (2S)-[2-2H1]-butan-2-ol formed from hydroxylation of the CH3CD2- end (0.012%), and the (2S)-[3-2H1]-butan-2-ol formed from hydroxylation of the monodeuterated butane (0.07%); and (iii) the 13C isotopomer of (2S)-[2-2H1]-butan-2-ol (0.048%). The total S-contribution without configurational inversion is thus 1.5%, which gives a S/R of 3.0%, in exact agreement with the observed S/R for the m/z 59 peaks in the GCMS. In other words, there is no evidence of configuration inversion at the secondary carbon during hydroxylation of the C-H bond in [2,2-2H3]butane.

The ester products arising from hydroxylation of the CH3CD2- end of [2,2-2H2]butane would contribute to the mass peaks at m/z 58. The R peak is dominated by (2R)-[2-2H1]-butan-2-ol from hydroxylation of prochiral CD2 center of the molecule (24%) and from hydroxylation of the monodeuterated butane at the C-H bond of the CH3CHD- end (4.5%); there is also a contribution of 9.1% arising from (2R)-[3-2H1]-butan-2-ol formed by hydroxylation of the prochiral CH2 center of the monodeuterated butane, and a small contribution (0.26%) from the 13C isoto- pomer of (2R)-butan-2-ol produced by oxidation of n-butane and the C-D in the background monodeuterated butane. The S peak includes contributions from: (a) (2S)-[2-2H1]-butan-2-ol (1.8%) from hydroxylation of the prochiral CD2 center of the [2,2-2H3]butane and hydroxylation of the monodeuterated butane at the C-H of the CH3CHD- end; (b) (2S)-[3-2H1]-butan-2-ol (1.1%) from hydroxylation of the prochiral CH2 center of the monodeuterated butane; and (c) the 13C isotopomers of (2R)- and (2S)-butan-2-ol (0.041%) from oxidation of the background n-butane. This analysis of the products yields a S/R of 7.6%, in excellent agreement with the observed upper limit of 7.6%. Thus, there is also no evidence of configurational inversion at the secondary carbon during hydroxylation of the C-D bond in this butane.

In summary, we conclude that the hydroxylation of [2,2-2H2]butane, (2S,3S)-[2-2H1,3-2H1]-butane, and (2R,3R)-[2-2H1-
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2H1,3-2H2-butane mediated by pMMO proceeds with full retention of configuration.

DISCUSSION

Taken together, the stereochemical findings summarized above are consistent with the competing substrate-binding modes proposed earlier, namely, the substrate butane can become hydroxylated by presentations of the C–H or C–D bonds on the Re and Si faces of the molecule to the active oxygen atom species at the active site for concerted oxo-transfer to the C–H or C–D bonds presented with absolute retention of the configuration at the carbon center attacked. The congestion of the active site in pMMO, including the surface roughness of the hydrophobic pocket, provides the van der Waals interactions required to discriminate between the two substrate orientations. This is the source of chiral selectivity that is observed here. In this study, we have exploited different C2/C3 chiral deuterated butanes and the H/D kinetic isotope effect to verify this picture. Thus, in the case of (2S,3S)-2-2H1,3-2H2-butane, the chiral selectivity of the hydroxylation is enhanced relative to that for normal butane, whereas it is significantly suppressed in the case of (2R,3R)-2-2H1,3-2H2-butane.

In the case of the (2R,3S*,3S*)-2-2H1,3-2H2-butane, two sets of 2-butanol diastereomers would be generated from hydroxylation on the Re and Si sites, one set from oxo-insertion into the C–H bond and the other, the C–D bond. Since the two sets of 2-butanol diastereomers contribute to the R- and S-stereoisomers to the same extent, there can be no isotope effect on the de ratio. Nevertheless, optical activity is induced by the hydroxylation process because of the chiral selectivity imposed on the substrate by the size and shape of the active site in the enzyme. This is analogous to the chiral selectivity previously reported for n-butane. Finally, from the throughputs of the [2-2H1,3-2H2]butan-2-ol versus [2-2H1,3-2H2]butan-2-ol and [3-2H1]butan-2-ol formed, we have obtained a kinetic isotope effect of 5.5 (2) on the hydroxylation rate (kH/kD).

In the case of [2,2-2H2]butane, the chiral selectivity is essentially totally biased toward the R-stereoisomer, irrespective of whether the hydroxylation occurs at the CH4 or CD4, center of the hydrocarbon molecule. There is also no deuterium isotope effect on the de ratio for this butane. This observation alone provides the strongest evidence in support of hydroxylation with total retention of configuration at the carbon center oxidized. We have also deduced a value of 5.5(0.8) for the kinetic isotope effect kH/kD from the throughputs of the [3,3-2H2]butan-2-ol and [2-2H2]butan-2-ol produced. Thus, except for the difference in the degree of chiral selectivity, the findings of the present study on the various butanes are in total congruence.

The experiments on [2,2-2H2]butane are intriguing in that the hydroxylation proceeds stereospecifically. This result underscores the remarkable variation in the free energy landscape for the deuterated butane in its various orientations within the hydrophobic pocket when the deuterium atoms are positioned over different secondary carbon centers. Clearly these effects reflect subtle variations in the van der Waals interactions between the hydrocarbon molecule and the walls of the hydrophobic pocket with the incorporation of deuterium atoms, and possibly, the ability of the pocket to modify its detailed shape and/or surface roughness to accommodate the different substrates. The stereochemical data that we have compiled for the various butane diastereomers indicate that the orientation of the CH3CH2 fragment within the hydrophobic pocket is sensitive to the nature of the deuterium substitution, perhaps, in response to the somewhat smaller van der Waals radius of the C–D bond compared with the C–H bond. Indeed, the smaller Km deduced for [2,2-2H2]butane relative to the other deuterated butanes indicates that [2,2-2H2]butane is by far the better substrate. The dramatic difference in free energy between the insertion of the CH3CH2 and the CH3CD2-ends of the [2,2-2H2]butane into the hydrophobic pocket (∼RTln(2.8)) is also consistent with this scenario. These observations for pMMO are quite unlike that for sMMO, where one has observed only a slight discrimination between the two diastereomers of the chiral [2-2H1]butanes in the hydroxylation chemistry (22). Presumably, there is a lack of strong steric constraints to orient the substrate within the active site of the sMMO enzyme.

Finally, the significant difference between Km deduced for [2,2-2H2]butane relative to the other deuterated butanes underscores the fact that deuterium substitution could have a very large isotope effect on Km in alkane oxidations mediated by the MMO enzymes. When large isotopic effects of this magnitude on the Km of the substrate butane isotopomers are not taken into consideration in the analysis of the kinetic turnovers of the alkane substrates, the determination of the kinetic isotope effects would be compromised. The large 1H/2H kinetic isotope reported by Lipscomb et al. (24) for the hydroxylation of CH4 and CD4 by sMMO was apparently in error because the authors neglected to include the large isotope effect on Km in the case of methane (Km is much larger for CD4 than for CH4) (25). For this reason, we have consistently compared the throughputs of the products derived from the deuterated chiral butanes with those expected for the non- and monodeuteriobutane in the same experiment to assure for sake of consistency that there is no significant variation in Km among the various substrate butane isotopomers. An exception was noted only for [2,2-2H2]butane. Since Km is substantially smaller for [2,2-2H2]butane compared with the other butanes, we would have obtained a significantly smaller kH/kD had we not included the isotope effect on Km in our analysis of the data.

Mechanistic Implications—The most important conclusion to emerge from the present study is that the hydroxylation chemistry mediated by pMMO must proceed with a mechanism that leads predominantly to retention of configuration. The hydroxylation of [2,2-2H2]butane yields butan-2-ol products that are totally biased toward the R-stereoisomer without evidence of any configurational inversion at the carbon center oxidized. In the case of the chiral deuterated butane experiments, we find that the stereoschemical distribution of the products obtained mirrors the stereochemical configuration of the various chiral substrates and their expected presentation to the active site for oxygen atom transfer, after allowance is made for the kinetic isotope effect on the oxo insertion. These findings, again, are only consistent with direct oxo insertion with full retention of configuration, as we have concluded here from detailed analysis of the GC-MS data.

The observed kinetic isotope effects for the hydroxylation chemistry do not seem to be consistent with the classical radical mechanism. If the hydroxylation proceeds via a direct concerted oxo insertion mechanism, the overall rate should be determined by the single oxo transfer step, as the on-off rates of the substrate as well as reorientations of the substrate to present the stereo heterotopic faces of C–H or C–D bonds to the hot oxygen atom or “O” species should be rapid. The relatively consistent kH/kD observed for the C–H and C–D bonds of the secondary carbons on the Re and Si faces of chiral deuterated butanes (−5), the intrinsic kH/kD of 5.5 determined for [2,2-2H2]butane, as well as the kH/kD observed for the primary carbon (5.2–5.5) in cryptically chiral ethanes, is not consistent with the end-on hydrogen-abstraction radical-rebound mechanism. Due to extensive hydrogen-tunneling in a linear transition state to form the hydroxyl radical, a much larger kH/kD,
would have been expected. Rather, these data are more in line with product formation from a non-linear early transition-state such as one that is formed from side-on “O” attack across a C–H or C–D bond, a scenario in which the electronic interactions between the C–H or C–D bond and the “O” atom would lead to a highly anharmonic potential energy surface for the transition state, possibly one that might even be double minimum in nature with a modest central barrier.

Recently, we propose a concerted mechanism for the oxo transfer step in the hydroxylation chemistry, in which an active oxene is delivered side-on from a bis-μ-dioxo-bridged Cu(II)-Cu(II) cluster to the C–O or C–D bond (25). This transition state could be a four- or five-centered one, depending on whether there is interaction between the metal center(s) delivering the oxene and the carbon center or the C–H bond accepting the hot “O” atom (Fig. 5). In any case, since the process is starting from a diamagnetic copper cluster, conservation of spin multiplicity dictates that the “O” atom be delivered as a singlet oxene. As shown in Fig. 5, the transient OH species that is moving away from the carbon would then be generated with the spin of the odd electron parallel to the odd spin of the electron localized on the carbon center. These two spins would thus be favorably aligned for rapid bond closure to form the C–O bond upon product formation. For such a scenario, there should be only a modest potential barrier to hinder the motion of the (H or D) atom during elongation of the C–H or C–D bond to form the final product in the transition state, and we would expect only a modest H/D kinetic isotope effect. Also, there should be only minor structural changes about the carbon center attacked during the formation of the transition state, in accordance with the observed lack of a 12C/13C kinetic isotope effect on the rate of the hydroxylation (25). This chemistry is analogous to the mechanism of singlet carbene insertion across C–H bonds, for which there has been a celebrated history (28).

In light of this discussion, the transition state involving the oxene of the copper cluster and the C–H bond of the alkane should be primarily singlet in character. This feature of the transition state promotes rapid bond closure, and the resultant process that ensues should be a concerted one. On the other hand, spin crossover into the triplet manifold could occur. Since the transition state is short-lived, no more than 10 femtoseconds, the amount of spin crossover could not be large. From the outcome of the present study, we could set an upper limit of 1–2% for any configurational inversion that might have occurred during the hydroxylation chemistry. We believe that any radicals that might have been responsible for configurational inversion of 1–2% should be construed as evidence for spin-crossover from the singlet to the triplet manifold in the transition state. In fact, with this added ambiguity, it would not be possible, in the case of [2,2-2H]butane, to distinguish between a configurational inversion of 1–2% due to a spin-crossover and a weak equilibrium (K°/RT ~ 0.02) for the presentation of the Re and Si faces of CH or CD bonds to the active site. This spin-crossover could also be the origin of the short-lived radical formed in the so-called concerted yet non-synchro-
nous radical mechanism advocated for the hydroxylation chemistry mediated by sMMO (22, 23).

Since the spin-crossover process is an irreversible one, the extent of branching or channeling of the reaction products (32) is limited by the extent of crossover. Accordingly, the amount of radicals formed during spin-crossover is determined by the lifetime of the transition state. However, once the system has crossed over to the triplet manifold, the fate of the radicals, or the outcome of the chemistry, is limited by the geminal recombination rate. This timescale for geminal recombination of radicals within the solvent cage is typically of the order of a few picoseconds, which is significantly longer. Geminal radical recombination is limited by the rate in which the radicals produced lose phase coherence, which in turn is influenced by the fluidity of the solvent cage in which the radicals are formed. For radicals within the hydrophobic pocket of a protein, the spin dynamics for the radicals to lose phase coherence could be quite long, about 1–10 ps, ample time for any reorientation or inversion process that must be invoked to describe them. As formulated, the concerted yet non-synchronous radical mechanism appears to be a ground-state process. Since the time span is significantly longer for such a ground state process (100 ps), many more radicals would be formed. Again, the fate of their chemistry must be determined by the geminal recombination rate of the radicals within the solvent cage. Thus, any ultrafast concerted yet non-synchronous process would be inconsistent with a ground state process. Only a concerted mechanism with spin-crossover from the singlet to the triplet manifold could account for the negligible amount of radicals formed in these reactions.

When spin-crossover does occur within the context of the concerted mechanism, C–O closure would not take place until the two spins are reverted back to the original singlet configuration. In the spin-correlated limit, however, any radicals formed should be short-lived, as suggested by radical clock experiments on the sMMO-mediated chemistry. When the C-center and OH-center radicals are sufficiently long-lived, one would not be able to distinguish between the concerted mechanism and the classical hydrogen-abstraction radical-rebound mechanism, except by the levels of radicals formed. Either way, the reaction remains, strictly speaking, concerted, as there is only one transition state involved in the overall chemistry. In any case, the scheme that we have described here unifies the two extreme mechanistic limits within the same overall-arched framework. Of course, had the “O” species been delivered in its triplet state, there would be no ambiguity in the interpretation. Under this circumstance, it would be more appropriate to invoke the classical hydrogen abstraction radical rebound mechanism, as in the case of cytochrome P450 chemistry.

Finally, aside from spin-crossover from the singlet to the triplet manifold in the transition state, crossover to an ionic state is also possible. Such a crossover could be responsible for the small amounts of rearrangement products detected in mechanistic probe experiments (27) that have been derived from a putative carboxylation precursor formed during the crossover.

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**Fig. 5.** Early transition state for side-on oxene insertion across a C–H bond.
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