The Stereoselectivity and Catalytic Properties of *Xanthobacter autotrophicus* 2-[(*R*)-2-Hydroxypropylthio]ethanesulfonate Dehydrogenase Are Controlled by Interactions between C-Terminal Arginine Residues and the Sulfonate of Coenzyme M†

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ABSTRACT: 2-[(*R*)-2-Hydroxypropylthio]ethanesulfonate (R-HPC) dehydrogenase (DH) catalyzes the reversible oxidation of R-HPC to 2-(2-ketopropylthio)ethanesulfonate (2-KPC) in a key reaction in the bacterial conversion of chiral epoxides to β-keto acids. R-HPCDH is highly specific for the *R*-enantiomer of HPC, while a separate enzyme, S-HPCDH, catalyzes the oxidation of the corresponding *S*-enantiomer. In the present study, the features of substrate and enzyme imparting stereospecificity have been investigated for R-HPCDH. S-HPC was a substrate for R-HPCDH with a K_m identical to that for R-HPC but with a k_{cat} 600 times lower. Achiral 2-propanol and short-chain (*R*)- and (*S*)-2-alkanols were substrates for R-HPCDH. For (*R*)-alkanols, as the carbon chain length increased, K_m decreased, with the K_m for (*R*)-2-octanol being 1700 times lower than for 2-propanol. At the same time, k_{cat} changed very little and was at least 90% lower than k_{cat} for R-HPC and at least 22 times higher than k_{cat} for S-HPC. (*S*)-2-Butanol and (*S*)-2-pentanol were substrates for R-HPCDH. The K_m for (*S*)-2-butanol was identical to that for (*R*)-2-butanol, while the K_m for (*S*)-2-pentanol was 7.5 times higher than for (*R*)-2-pentanol. Longer chain (*S*)-2-alkanols were sufficiently poor substrates for R-HPCDH that kinetic parameters could not be determined. Mutagenesis of C-terminal arginine residues of R-HPCDH revealed that R152 and R196 are essential for effective catalysis with the natural substrates R-HPC and 2-KPC but not for catalysis with 2-alkanols or ketones as substrates. Short-chain alkylsulfonates and coenzyme M (2-mercaptoethanesulfonate) were found to modify the kinetic parameters for 2-butanone reduction by R-HPCDH in a saturable fashion, with the general effect of increasing k_{cat}, decreasing K_m, and increasing the enantioselectivity of 2-butanone reduction to a theoretical value of 100% (*S*)-2-butanol. The modulating effects of ethanesulfonate and propanesulfonate provided thermodynamic binding constants close to K_m for the natural substrates R-HPC and 2-KPC. The effects of alkylsulfonates on modulating the enantioselectivity and kinetic properties of R-HPCDH were abolished in R152A and R196A mutants but not in mutants of other C-terminal arginine residues. Collectively, the results suggest that interactions between the sulfonate of CoM and specific arginine residues are key to the enantioselectivity and catalytic efficiency of R-HPCDH. A model is proposed wherein sulfonate−arginine interactions within an alkylsulfonate binding pocket control the catalytic properties of R-HPCDH.

Enzymes that interconvert alcohol and carbonyl groups often exhibit a high degree of stereoselectivity for chiral alcohol production and utilization. Accordingly, alcohol dehydrogenases have been studied extensively both in the contexts of elucidating the features that control substrate specificity and for utilization as biocatalysts in stereoselective synthesis (*1*). With regard to stereoselective synthesis, a number of strategies have been employed to improve or alter the enantioselectivity of alcohol dehydrogenation and other chiral transformations (*2*, *3*). In the case of purified enzymes, these strategies include traditional protein engineering, enzyme screening, directed evolution, and variations in assay conditions, e.g., temperature or solvent (*4*, *5*). While all of these approaches have enjoyed various degrees of success, none of these strategies is broadly applicable, and accordingly, new methods for altering enzyme stereoselectivity continue to be explored.

In the present work, a new method has been identified for altering enzyme stereoselectivity. This method, which employs linear alkylsulfonates to alter the stereoselectivity of 2-butanone reduction, emerged during the course of our investigation into the mechanism of substrate binding and stereoselectivity of a novel bacterial alcohol dehydrogenase. This dehydrogenase, designated 2-[(*R*)-2-hydroxypropylthio]-ethanesulfonate dehydrogenase (R-HPCDH,[1] EC 1.1.1.268), is an integral enzyme in the pathway of propylene metabolism in *Xanthobacter autotrophicus* strain Py2 (*6*−*9*). In this pathway, propylene is converted into a 95:5 enantiomeric

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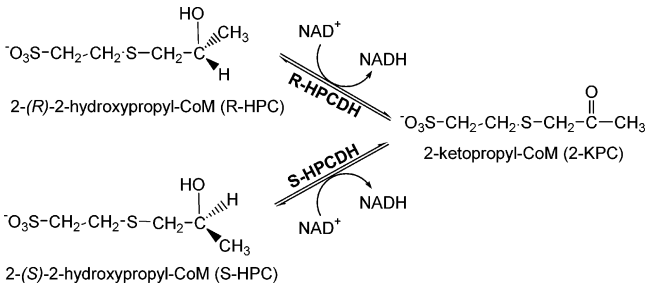
Scheme 1



mixture of (*R*)- and (*S*)-epoxypropane via an alkene monooxygenase (*10*). Enantiomers of epoxypropane are subsequently ring opened by the thiol of the atypical cofactor coenzyme M (CoM) in a reaction catalyzed by a zinc-dependent epoxyalkane:CoM transferase (*6*, *8*, *11*). The resulting products of this reaction are R- and S-HPC, and these molecules are the substrates for the R-HPCDH and S-HPCDH enzymes, respectively (Scheme 1) (*8*). The R- and S-HPC dehydrogenases are homologous enzymes belonging to the short-chain dehydrogenase/reductase (SDR) family of enzymes (*7*). They both catalyze the NAD^+-dependent oxidation of HPC but exhibit a high degree of stereoselectivity for their respective enantiomers (Scheme 1) (*8*, *9*). The product of R- and S-HPC oxidation is 2-KPC, which is subsequently cleaved and carboxylated in a reductant-dependent reaction, releasing free CoM and acetoacetate (*12*, *13*).

Studies of R-HPCDH have provided insights into the kinetic and catalytic mechanism of this enzyme (*9*). Specifically, the reaction was shown to proceed by a compulsory ordered mechanism, the identity and function of catalytic residues was established, and chemical modification studies suggested that one or more arginine residues are important in substrate binding, presumably by interacting with the sulfonate of CoM (*9*). Studies of S-HPCDH have lagged behind those of R-HPCDH due to difficulties encountered in expressing an active form of the enzyme in heterologous expression systems (*9*), but the same general principles are believed to apply to the catalytic mechanism of this enzyme.

Of central importance to the present work, key residues within the N-terminal and central domains, which contain the NAD^+ binding and catalytic residues, are conserved for all SDR enzymes, while differences in the C-terminal domains confer specificity for the enzyme substrate (*14*, *15*). R- and S-HPCDH have a number of differences in amino acid sequence in their respective C-terminal domains, most striking being the difference in placement of arginine residues that may play a role in binding of the sulfonate of CoM through salt bridges (*16*). A model for substrate binding and catalysis has been proposed in which differential placement of charged residues is responsible for proper binding of R-HPC and S-HPC to the respective enzymes (*12*, *16*). As shown in Scheme 2, differential placement of positively charged arginine residues within the C-terminal substrate binding domains of the enzymes could provide the "switch" necessary for this chiral discrimination. This hypothesis has been investigated by examining the catalytic properties and stereoselectivity of native and site-directed mutants of R-HPCDH with the physiological substrates R-HPC and 2-KPC and aliphatic alcohols and ketones lacking the sulfonate moiety. The effects of linear alkylsulfonates on the kinetics and stereoselectivity of aliphatic ketone reduction were also investigated. The results support a model where specific sulfonate−arginine interactions dictate the catalytic properties and stereospecificity of R-HPCDH.

EXPERIMENTAL PROCEDURES

Materials. Commercially available organic compounds were purchased from Sigma-Aldrich Chemicals. 2-(2-Ketopropylthio)ethanesulfonate (2-KPC) and HPC enantiomers were synthesized as described previously (*13*).

Media and Growth of Bacteria. All procedures were performed as described previously (*9*).

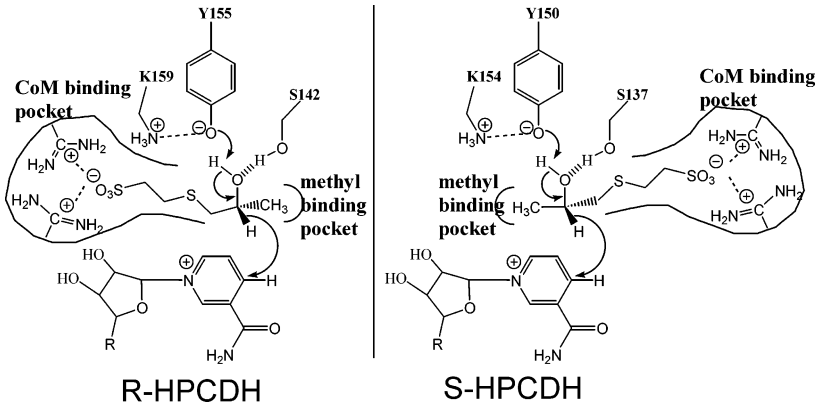
Protein Engineering. All oligonucleotides utilized for constructing site-specific mutations were purchased from MWG Biotech. Site-directed mutagenesis of pXD28 was carried out utilizing the Quickchange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocols. All mutations were confirmed by DNA sequencing. The sequences of the mutagenic primer pairs utilized for each codon substitution in *xecD* are as follows: R152A, GTCGCGTTCCCGGGGGCCTCCGCCTACACCACC and GGTGGTGTAGGCGGAGGCCCCCGGGAACGCGAC; R179A, GCCGGCTCCGGCATCGCCTGCAACGCGGTCTGT and ACAGACCGCGTTGCAGGCGATGCCGGAGCCGGC; R196A, ATGACCCAGTGGGCCCTCGACCAGCCGGAG and CTCCGGCTGGTCGAGGGCCCACTGGGTCAT; R203A, GACCAGCCGGAGCTGGCCGACCAGGTCCTGGCC and GGCCAGGACCTGGTCGGCCAGCTCCGGCTGGTC; R209A, CAGGTCCTGGCCGCCATCCCGCAAAAGGAG and CTCCTTTTGCGGGATGGCGGCGAGGACCTG.

DNA Sequencing. DNA sequencing was performed on PE/ABI 377 and 373A stretch sequencers with Taq FS terminator chemistry at the Utah State University Biotechnology Center DNA Sequencing Laboratory. The following sequencing primer (designated primer 4203) was used to confirm pXD28 mutants R152A and R179A: ACGCCCGTCGAGCAGTTCGACAAG. The following sequencing primer (designated primer 4420) was used to confirm pXD28 mutants R196A, R203A, and R209A: CCGGCTCCGGCATCCGCTGCAACG.

Purification of rR-HPCDH and rR-HPCDH Mutants and Protein Characterization. The purification of rR-HPCDH proteins was performed as described previously (*9*). SDS−PAGE, native PAGE, gel filtration chromatography, and CD spectrapolarimetric analyses were performed on all rR-HPCDH proteins to verify the integrity of mutant enzymes, using the procedures described previously (*9*). All of the mutant enzymes described herein behaved as dimers and had CD spectra indistinguishable from wild-type rR-HPCDH.

[1] Abbreviations: CoA, coenzyme A; CoB, coenzyme B (7-thioheptanoylthreonine phosphate); CoM, coenzyme M (2-mercaptoethanesulfonate); CD, circular dichroism; EC, enzyme classification; EE, enantiomeric excess; R-HPC, 2-[(*R*)-2-hydroxypropylthio]ethanesulfonate [2-(*R*)-2-hydroxypropyl-CoM]; S-HPC, 2-[(*S*)-2-hydroxypropylthio]ethanesulfonate [2-(*S*)-2-hydroxypropyl-CoM]; S-HPCDH, 2-[(*S*)-2-hydroxypropylthio]ethanesulfonate dehydrogenase; R-HPCDH, 2-[(*R*)-2-hydroxypropylthio]ethanesulfonate dehydrogenase; rR-HPCDH, recombinant 2-[(*R*)-2-hydroxypropylthio]ethanesulfonate dehydrogenase; 2-KPC, 2-(2-ketopropylthio)ethanesulfonate (2-ketopropyl-CoM); 2-KPCC, 2-(2-ketopropylthio)ethanesulfonate carboxylase/oxidoreductase; MCR, methyl-coenzyme M reductase; SDR, short-chain dehydrogenase/reductase; Tris, tris(hydroxymethyl)aminomethane.

Scheme 2



Spectrophotometric Enzyme Assays and Data Analysis. All enzyme assays were performed in 50 mM glycine, 50 mM NaH_2PO_4, and 50 mM Tris base (GPT buffer mix) at a pH of 7.5. This buffer formulation was shown previously to give optimal R-HPCDH activity over a range of pH values (*9*). Goode buffers are inhibitory to R-HPCDH, possibly due to the presence of the sulfonate functional group, and they have purposefully been avoided for studies of R-HPCDH. Assays were performed at 30 °C in a Shimadzu model UV160U spectrophotometer containing a water-jacketed cell holder for thermal control. Assays were initiated by the addition of enzyme. The ΔA_{340nm}·min^{-1} was correlated with either micromoles of alcohol oxidized or ketone reduced using the extinction coefficient for NADH ($\epsilon_{340} = 6.22$ mM^{-1}·cm^{-1}). For assays of alcohol oxidation, the following ranges of alcohol concentrations were used in determining kinetic constants: R-HPC, 0.025−1.0 mM; S-HPC, 0.050−1.7 mM; 2-propanol, 100−4600 mM; (*R*)- and (*S*)-2-butanol, 50−1300 mM; (*R*)-2-pentanol, 4.6−185 mM; (*S*)-2-pentanol, 20−370 mM; (*R*)-2-hexanol, 2.4−24 mM; (*R*)-2-heptanol, 1.0−16 mM; (*R*)-2-octanol, 0.25−5 mM. The concentration of NAD^+ for all assays was 10 mM, a value that is 26 times higher than K_m (*9*). For assays of ketone reduction, the following concentration ranges were used in determining kinetic constants: 2-KPC, 0.025−1.0 mM; 2-butanone, 10−200 mM. The concentration of NADH for these assays was 0.16 mM, a value 4.4 times higher than K_m (*9*). Typically, seven concentrations of substrates within the ranges indicated were chosen for the kinetic analyses. All assays were performed in triplicate. Kinetic constants (K_m and V_{max}) and standard errors were calculated by fitting initial rate data to the standard form of the Michaelis−Menten equation using the software SIGMAPLOT. Since the concentrations of NADH and NAD^+ in all assays were fixed, and not varied concomitant with the organic substrate as is done for a true bisubstrate kinetic analysis (*9*), the kinetic parameters should technically be referred to as "apparent K_m and apparent V_{max}".

Chiral Gas Chromatographic Assay for 2-Butanone Reduction. Assays were performed in crimp-sealed 3 mL serum vials containing 1 mL total volumes. Assay components were NADH (15 mM), 2-butanone (56 mM), and 0.64 mg of enzyme (wild-type or mutant R-HPCDH) in GPT buffer mix, pH 7.5, containing 15% (v/v) glycerol. Assay vials were allowed to shake (200 rpm) in a 30 °C water bath for 1 h before 250 μL of headspace gas was removed and injected into a Shimadzu GC-8A gas chromatograph outfitted with a Supelco β-Dex 225 (30 m × 0.53 mm) column. Gas chromatography was performed with nitrogen as the carrier gas and with the following parameters: injector temperature, 200 °C; column temperature, 50 °C. Under these conditions enantiomers of 2-butanol were efficiently resolved, and (*S*)-2-butanol eluted first.

RESULTS

R-HPCDH Specificity for the R-Enantiomer of HPC Is Dictated by k_{cat}, not K_m. R-HPCDH and S-HPCDH are highly specific for their respective substrates (R-HPC and S-HPC), exhibiting less than 0.5% activity when the opposite enantiomer is used as the substrate (*8*). In a previous study in which the active site catalytic triad of R-HPCDH was defined and the enzyme was kinetically characterized (*9*), the enzyme was reported to have $K_m = 105$ μM and $k_{cat} = 26$ s^{-1} with the physiological substrate R-HPC. S-HPC was shown to be a competitive inhibitor of R-HPC oxidation with $K_i = 156$ μM, suggesting that it binds to R-HPCDH with an affinity comparable to that of the natural substrate (*9*). This has now been confirmed by kinetically characterizing S-HPC as a substrate for R-HPCDH. In side by side experiments with R-HPC and S-HPC as substrates, the kinetic parameters reported in the first two rows of Table 1 were obtained. The apparent K_m values for the two substrates are indistinguishable, while the apparent k_{cat} values differ by 600-fold. Together with the inhibition results reported earlier (*9*), it can be concluded that both enantiomers of HPC bind to R-HPCDH with comparable affinities. Using the model presented in Scheme 2 as a reference, the ethanesulfonate moiety of HPC is probably the primary determinant in the formation of the ES complex. Accordingly, the lower activity with S-HPC as the substrate presumably arises from the necessary misalignment of (i) the hydroxyl group, from which H^+ must be removed by the general base Y155, and (ii) the hydrogen atom on the alcoholic carbon atom, which should be aligned properly with NAD^+ for hydride abstraction and transfer to NAD^+. The low rate of turnover with S-HPC presumably results from the formation of an ES complex that is intermediate between these extremes, wherein the hydroxyl group and hydride are oriented sufficiently properly to allow slow turnover.

Aliphatic Alcohols as Substrates for R-HPCDH. Previously, R-HPCDH was shown to have some catalytic activity with short-chain secondary aliphatic alcohols, although very high concentrations of the alcohols were required to give

Table 1: Kinetic Parameters for R-HPCDH with Various Secondary Alcohols at 30 °C and pH 7.5[a]

secondary alcohol	V_{max} (units·mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	enantio-selectivity (*E*)
R-HPC	56.8 ± 0.5	0.102 ± 0.002	26.8	2.6×10^5	
S-HPC	0.093 ± 0.001	0.100 ± 0.001	0.044	4.4×10^2	590
2-propanol	6.2 ± 0.1	1726 ± 88	2.9	1.7×10^0	NA[b]
(*R*)-2-butanol	2.07 ± 0.05	328 ± 18	0.98	3.0×10^0	
(*S*)-2-butanol	4.72 ± 0.04	315 ± 11	2.2	7.1×10^0	0.42
(*R*)-2-pentanol	2.36 ± 0.03	20 ± 1	1.1	5.6×10^1	
(*S*)-2-pentanol	6.19 ± 0.17	153 ± 8	2.9	1.9×10^1	2.9
(*R*)-2-hexanol	5.66 ± 0.03	4.6 ± 0.2	2.7	5.8×10^2	
(*S*)-2-hexanol	ND[c]	ND	ND	ND	ND
(*R*)-2-heptanol	3.80 ± 0.03	2.64 ± 0.03	1.8	6.8×10^2	
(*S*)-2-heptanol	ND	ND	ND	ND	ND
(*R*)-2-octanol	3.54 ± 0.13	1.08 ± 0.08	1.7	1.5×10^3	
(*S*)-2-octanol	ND	ND	ND	ND	ND

[a] Assays utilizing R-HPC and S-HPC contained 4 and 320 μg of enzyme, respectively. Assays for all other secondary alcohols contained 100 μg of enzyme. Enantioselectivity was defined as $(k_{cat}/K_m)_{R-enantiomer}/(k_{cat}/K_m)_{S-enantiomer}$. Apparent V_{max} and K_m values are reported as means ± standard deviations. All other values are reported as means only. [b] NA, not applicable; 2-propanol is achiral. [c] ND, not determined; accurate steady-state kinetic data were unobtainable due to lack of saturation up to the point of substrate solubility.

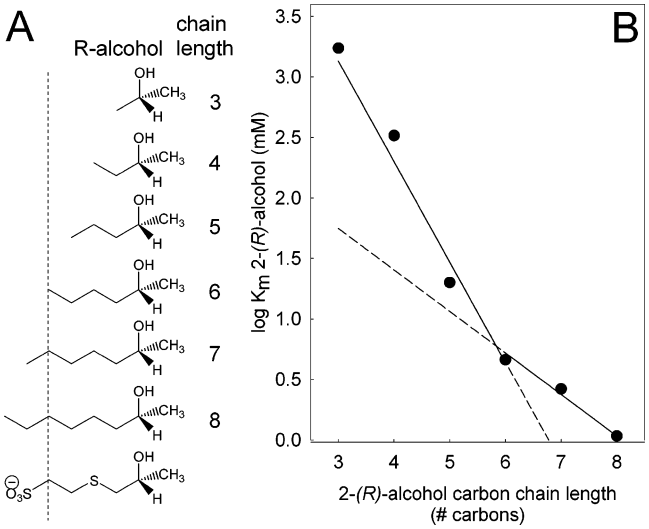


FIGURE 1: Relative affinity (K_m) of R-HPCDH for 2-propanol and chiral (*R*)-2-alcohols as a function of carbon chain length. Panel A: Structures of (*R*)-2-alcohols. Panel B: Semilog plot of K_m vs (*R*)-2-alkanol carbon chain length. The two lines were from logarithmic fits of the experimental data, using chain lengths 3−6 and 6−8 for the fits. The solid lines extend through the data points; the dashed lines are extrapolations beyond the data points.

rates much lower than that observed with R-HPC (*9*). This observation has now been extended to kinetically characterize the oxidation of a range of secondary aliphatic alcohols ranging from the achiral 2-propanol to the *R*- and *S*-enantiomers of the C4 to C8 2-alkanols (Table 1). With regard to the (*R*)-alcohols, K_m values decreased as the carbon chain length was increased, with a 1700-fold decrease in K_m upon going from 2-propanol to (*R*)-2-octanol (Table 1). At the same time, k_{cat} values remained fairly constant and were 10−30 times lower than k_{cat} for R-HPC and 22−67 times higher than k_{cat} for S-HPC (Table 1). These results demonstrate that carbon chain length is not a major determinant in turnover rate, which is presumably dictated by proper alignment of the hydroxyl group and hydrogen atom for general base abstraction and hydride transfer.

The relationship between K_m and carbon chain length for the (*R*)-2-alkanols is represented graphically in Figure 1. There is a linear decrease in log K_m with increasing carbon chain length up to (*R*)-2-hexanol, the alkanol that has the same chain length as the natural substrate R-HPC (neglecting the terminal sulfonate). At this point, K_m continues to decrease linearly from (*R*)-2-hexanol to (*R*)-2-octanol but with a less negative slope. It is intriguing that this "break point" for slope difference occurs at the chain length representing the transition from "shorter than" to "longer than" the natural substrate 2-HPC (Figure 1B).

The *S*-enantiomers of 2-butanol and 2-pentanol were substrates for R-HPC, while the longer chain (*S*)-2-alkanols were sufficiently poor substrates that kinetic constants could not be determined. As shown in Table 1, (*R*)-2- and (*S*)-2-butanol have K_m values that are statistically identical, while the K_m for (*S*)-2-pentanol increases dramatically relative to the *R*-enantiomer (7.5-fold). Together, these results suggest that enantiomers of 2-butanol bind with high affinity with either the methyl or ethyl group (relative to the alcoholic carbon) oriented in the CoM binding pocket, while enantiomers of 2-pentanol bind 7.5 times better with the propyl group in the CoM binding pocket relative to the methyl. Apparently, chain lengths beyond propyl cannot be accommodated in the "methyl binding pocket", as reflected by the inability of (*S*)-2-hexanol, (*S*)-*2*-heptanol, and (*S*)-*2*-octanol to serve as effective substrates. At this point, 2-butanol is the most interesting of the aliphatic alcohols that are substrates for R-HPC, given the identical K_m values for the *R*- and *S*-enantiomers.

Role of C-Terminal Arginine Residues in Substrate Binding and Catalysis by R-HPCDH. Chemical modification studies suggested that one or more arginine residues may play a role in binding the sulfonate of R-HPC (*9*). The C-terminal domains of SDR enzymes are responsible for imparting substrate specificity, and there are striking differences in the placement of arginine residues within the C-terminal domains of S-HPCDH and R-HPCDH (*16*). Therefore, the expression system developed for R-HPCDH was exploited to mutagenize the C-terminal arginine residues, and the resultant mutant enzymes were purified and characterized.

The recombinant wild-type enzyme and all five of the C-terminal arginine mutants (R to A) were found to have similar banding profiles on SDS−PAGE and native PAGE and elution profiles on gel filtration chromatography, indicating that the native α_2 subunit structures are intact for each mutant. In addition, the CD spectra of the enzymes were

Table 2: Kinetic Parameters for the C-Terminal Arginine Mutants of R-HPCDH with Respect to 2-KPC and 2-Butanone Reduction[a]

	ketone substrate							
	2-KPC				2-butanone			
enzyme	V_{max} (units·mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	V_{max} (units·mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
WT	51.9 ± 0.5	0.092 ± 0.003	24.5	2.7×10^5	0.058 ± 0.002	52 ± 4	0.027	0.52
R152A	~ 2.9[b]	~ 27[b]	1.4	52	0.059 ± 0.001	41 ± 3	0.028	0.68
R179A	ND[c]	ND			ND	ND		
R196A	~ 5.0[b]	~ 8.7[b]	2.4	270	0.069 ± 0.001	23 ± 1	0.033	1.4
R203A	59.2 ± 0.5	0.44 ± 0.010	27.9	6.3×10^4	0.026 ± 0.001	39 ± 1	0.012	0.31
R209A	32 ± 2	0.15 ± 0.005	15	1.0×10^5	0.068 ± 0.002	84 ± 3	0.032	0.38

[a] Assays for 2-KPC reduction contained 4 μg of enzyme while assays for 2-butanone reduction contained 100 μg of enzyme. Apparent V_{max} and K_m values are reported as means ± standard deviations. All other values are given as means only. [b] The estimated K_m values for 2-KPC for these mutants were well above the [2-KPC] that could be added to assays due to the low solubility of 2-KPC. Thus, the values of K_m and V_{max} obtained by fitting the experimental data should be considered as estimates of the values. [c] ND, no detectable activity.

indistinguishable, indicating that no major structural changes resulted from the mutations.

The C-terminal arginine mutants were kinetically characterized relative to the recombinant wild-type enzyme with respect to two activities: reduction of 2-KPC to HPC and reduction of 2-butanone to 2-butanol. R-HPCDH has already been shown to be freely reversible with respect to R-HPC oxidation and 2-KPC reduction, and the k_{cat} and K_m values are very similar for both directions (*9*). The reverse reactions were chosen for the analyses because, for initial characterization, there was no need to consider the complicating factor of chirality of the starting substrate and because the kinetic characterization of the reverse reactions is central to the subsequent studies involving the addition of enantioselective modulators.

The results of these kinetic analyses are presented in Table 2. Two mutants, R152A and R196A, are especially noteworthy, as substantially different effects on kinetic parameters are observed for the natural substrate 2-KPC versus the effects seen for 2-butanone (relative to the wild-type enzyme). Specifically, the k_{cat} values for these two mutants were decreased substantially for the natural substrate 2-KPC, while the K_m values for 2-KPC were increased by roughly 100−300-fold. The same mutants, however, had kinetic parameters with respect to 2-butanone reduction that were changed very little vs the wild-type enzyme (Table 2). These results demonstrate that R152 and R196 are important for catalysis with the natural substrate but are of little importance with a neutral aliphatic ketone as substrate. The obvious interpretation is that R152 and R196 play a key role in the binding of the sulfonate of the natural substrate. Of the remaining three mutants, the R179A mutant was inactive for all activities, suggesting that this residue is essential for catalysis. The R203A mutant had a k_{cat} for 2-KPC reduction identical to that of the wild-type enzyme, while the K_m value was increased by 5-fold (Table 2). While this is a significant increase in K_m, it is much smaller than the 100−300-fold higher K_m values for the R152A and R196A mutants and the even greater decrease in catalytic efficiency for the latter mutants (Table 2). The final mutant, R209A, had catalytic properties that were altered very little with respect to the wild-type enzyme. Collectively, the results of the kinetic analyses of 2-KPC and 2-butanone reduction presented in Table 2 lead us to conclude that R152 and R196 play a crucial role in binding the sulfonate of the natural substrate 2-KPC.

Enantioselective Modulators of 2-Butanone Reduction. The results presented above suggest that a binding pocket for the alkylsulfonate moiety of the substrate is crucial to the high degree of stereospecificity exhibited by R-HPCDH. In considering the data presented in Tables 1 and 2 and Figure 1, and the model for the R-HPCDH active site proposed in Scheme 2, it occurred to us that the stereospecificity of 2-butanone reduction might be modulated by including a separate and nonreactive "effector molecule" containing the alkylsulfonate moiety of the natural substrate. To form both (*R*)-2- or (*S*)-2-butanol as products, 2-butanone must bind at the active site of R-HPCDH in two different orientations, i.e., with the spatial orientations of the methyl and ethyl groups reversed. Accordingly, the two orientations will result in hydride transfer from NADH to different faces of the sp^2-hybridized carbonyl, resulting in formation of either (*R*)-2- or (*S*)-2-butanol as the product. The rationale behind the inclusion of a separate effector molecule is that, by binding in the CoM binding pocket, it may favor the binding of 2-butanone in one of these two orientations, and/or otherwise alter the catalytic properties of the enzyme, resulting in a difference in the ratio of chiral alcohol production.

For these analyses, R-HPCDH was incubated with 2-butanone and NADH for a sufficient time period to produce approximately 1 mM alcohol from 56 mM 2-butanone (based on rates with no effector present). At that time, the yields of (*R*)- and (*S*)-2-butanol enantiomers were determined by chiral gas chromatography. As shown in Table 3, R-HPCDH produced 70% (*S*)-2-butanol and 30% (*R*)-2-butanol under the experimental conditions in the absence of additives. This result agrees well with the data presented in Table 1, where the rate of (*S*)-2-butanol oxidation to 2-butanone is reported to be twice as fast as the rate of (*R*)-2-butanol oxidation. Thus, the approximate 2:1 selectivity for *S*:*R* 2-butanol holds in either the forward or reverse direction.

As shown in Table 3, the addition of ethanesulfonate or CoM to assays resulted in a dramatic increase in the yield of (*S*)-2-butanol relative to (*R*)-2-butanol. This modulator effect was highly specific for alkylsulfonates: two carboxylates tested, propionate and acetate, had little if any effect on the product distribution (Table 3). Likewise, ethylamine, ethanol, inorganic sulfate ion, inorganic phosphate ion (a component of the assay buffer; it is not inhibitory nor does it alter the catalytic properties of R-HPCDH), and sodium chloride had no effect on the product distributions (Table 3).

Table 3: Additives Tested for Their Ability To Modify the Enantioselectivity of R-HPCDH-Catalyzed 2-Butanone Reduction[a]

additive	chiral GC analysis following asymmetric reduction of 2-butanone			ΔEE (*S*)-2-butanol vs none
	% (*S*)-2-butanol	% (*R*)-2-butanol	EE (*S*)-2-butanol	
none	69.8 ± 0.9	30.2 ± 0.9	39.6	0
$CH_3CH_2SO_3^-$ Na^+	95.4 ± 0.3	4.6 ± 0.3	90.8	51.2
$HSCH_2CH_2SO_3^-$ Na^+	93.9 ± 0.3	6.1 ± 0.3	87.8	48.2
$CH_3CH_2COO^-$ Na^+	76.0 ± 0.4	24.0 ± 0.4	52.0	12.4
CH_3COO^- Na^+	73.3 ± 0.3	26.7 ± 0.3	46.6	7.0
$CH_3CH_2NH_3^+$ Cl^-	72.4 ± 0.6	27.6 ± 0.6	44.8	5.2
CH_3CH_2OH	72.7 ± 0.2	27.3 ± 0.2	45.4	5.8
Na_2SO_4	72.9 ± 0.4	27.1 ± 0.4	45.8	6.2
NaCl	70.2 ± 0.3	29.8 ± 0.3	40.4	0.8

[a] All additives were present at an overall concentration of 1 mM. All assays were performed in triplicate at 30 °C using 0.64 mg of R-HPCDH, NADH (15 mM), and 2-butanone (56 mM). Percent yields are reported as means ± standard deviations.

The concentration dependence of the C1 to C4 linear alkysulfonates on this phenomenon of "enantioselective modulation" was investigated. As shown in Figure 2A, in the presence of a fixed concentration of 2-butanone, the percentage of (*S*)-2-butanol increased in a hyperbolic and saturable fashion as the concentration of alkylsulfonate added to the assay was increased. The data in Figure 2A were fit to the equation for a rectangular hyperbola, in which a y_0 term was incorporated to account for the nonzero *y*-intercept:

$$\%\ \mathrm{SB} = \%\ \mathrm{SB}_0 + \frac{\%\ \mathrm{ISB}_{max}[\mathrm{AS}]}{K_{mod} + [\mathrm{AS}]} \qquad (1)$$

In this equation, % SB is the percentage of (*S*)-2-butanol formed, % SB_0 is the percentage of (*S*)-2-butanol formed in the absence of an effector, % ISB_{max} is the theoretical maximal increase in concentration of (*S*)-2-butanol at the saturation point, [AS] is the concentration of alkylsulfonate added to the assay, and K_{mod} is the activation (modulation) constant for the alkylsulfonate.

The data for each of the four alkylsulfonates fit very well to this equation (the lines in Figure 2A are derived from the curve fits). The K_{mod} terms were determined to be as follows: methanesulfonate, 286 ± 23 μM; ethanesulfonate, 157 ± 7.4 μM; propanesulfonate, 209 ± 21 μM; and butanesulfonate, 872 ± 77 μM. Thus, ethanesulfonate binds with the highest affinity to the enzyme, followed by propanesulfonate. By way of comparison, the K_m value for the natural substrate 2-KPC is 92 μM, while the K_m for 2-butanone is orders of magnitude higher (52 mM, Table 2). Thus, ethanesulfonate and propanesulfonate bind to R-HPCDH with affinities comparable to that of the natural substrate 2-KPC, providing further evidence that the major determinant in binding of substrate is the interaction of the ethanesulfonate side chain with the CoM binding pocket. The theoretical maximal yields of (*S*)-2-butanol with methanesulfonate, ethanesulfonate, and propanesulfonate were all within 1% of 100% (99.2−100.1% range) when % ISB_{max} was added to % SB_0 for each fit, demonstrating that the yield of (*S*)-2-butanol will reach 100% at the saturation point with these effectors. For butanesulfonate, a theoretical maximal yield of 92% was determined.

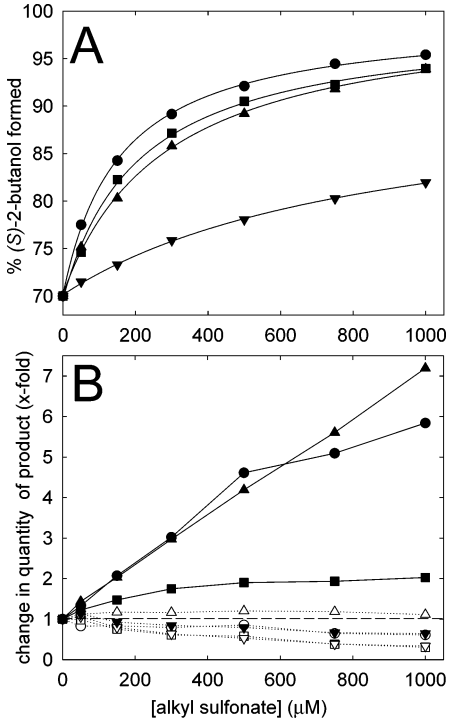


FIGURE 2: Linear alkylsulfonates as enantioselective modifiers of 2-butanone reduction. (A) Effect of alkylsulfonate concentration on percentage of (*S*)-2-butanol formed in fixed time assays. The alkylsulfonates present were (▲) methanesulfonate, (●) ethanesulfonate, (■) propanesulfonate, and (▼) butanesulfonate. (B) Effect of alkylsulfonate concentration on the relative amount of (*R*)-2-butanol and (*S*)-2-butanol formed in the fixed time assays. The change in quantity of product (*x*-fold) is relative to assays without an alkylsulfonate present. Quantities less than one reflect a net decrease in enantiomer production relative to the control. Closed (darkened) symbols represent (*S*)-2-butanol while the open symbols represent (*S*)-2-butanol. The identity of the symbols is the same as for (A).

The effect of the four alkylsulfonates on 2-butanol yields was further investigated by quantifying the absolute amount of (*R*)-2-butanol and (*S*)-2-butanol formed at the conclusion of the fixed time point assays. Interestingly, the total amount of the *S*-enantiomer increased dramatically (up to 7-fold more) in the assays containing methanesulfonate or ethanesulfonate (Figure 2B). At the same time, the amount of *R*-enantiomer increased slightly with methanesulfonate present but decreased slightly with ethanesulfonate present (Figure 2B). With propanesulfonate as the effector, the amount of *S*-enantiomer increased much less dramatically (less than 2-fold) while the amount of *R*-enantiomer decreased even more than for ethanesulfonate (Figure 2B). Finally, the yields of (*S*)-2- and (*R*)-2-butanol both decreased in the assays with butanesulfonate present but in such a manner to give the overall distributions presented in Figure 2A.

This modulating phenomenon was further investigated by determining the kinetic parameters for 2-butanone reduction in the presence of fixed (1 mM) concentrations of the four alkylsulfonates. As shown in Figure 3, Michaelis−Menten kinetics were observed for 2-butanone reduction in the presence of the various alkylsulfonates, but the kinetic parameters were altered by the presence of the effectors. Both k_{cat} and K_m were modulated by the alkylsulfonates (Table 4). A general trend was observed: the presence of methanesulfonate and ethanesulfonate resulted in a decrease in

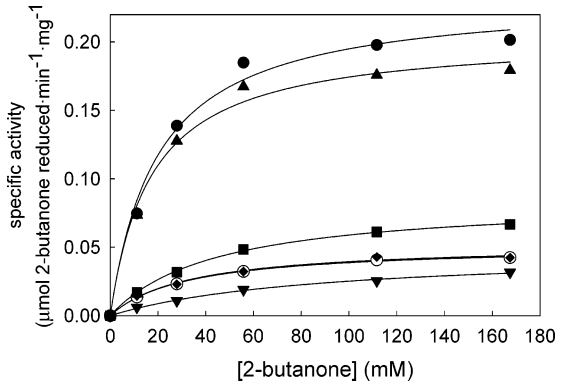


FIGURE 3: Effect of linear alkylsulfonates on the kinetic parameters of 2-butanone reduction by R-HPCDH. All additives were present at 1 mM concentrations. Symbols: (▲) methanesulfonate, (●) ethanesulfonate, (■) propanesulfonate, (▼) butanesulfonate, (◆) sodium sulfate, and (○) no additive present.

K_m and increase in k_{cat} relative to no effector, resulting in cooperative effects that served to raise the catalytic efficiency by about 11-fold. As the alkyl chain length was increased to C3 and C4, this effect reversed, with K_m increasing and then exceeding the control and k_{cat} decreasing simultaneously (Table 4).

How the presence of alkylsulfonates modulates both K_m and k_{cat} is at present unclear. The data support a model in which the alkylsulfonate binds in the CoM binding pocket with affinity comparable to that of the natural substrate 2-KPC. The presence of the alkylsulfonate effector influences both the orientation in which 2-butanone binds and the rate at which the bound substrate reacts to form product, resulting in the overall effects presented in Figures 2 and 3 and Tables 3 and 4. Focusing on ethanesulfonate as the effector, Scheme 3 proposes that the presence of ethanesulfonate results in 2-butanone binding preferentially in the orientation that leads to the formation of (*S*)-2-butanol as the product. Steric clashes between the bound alkylsulfonate and the ethyl side chain of 2-butanone when it binds in the orientation that would result in (*R*)-2-butanol formation might be responsible for favoring the former orientation. In addition to influencing the orientation of 2-butanone binding, perhaps the alkylsulfonate induces a conformational change at the active site that results in increased activity with 2-butanone bound in the preferred conformation, for example, by bringing NADH and Y155 in proper proximity for reaction.

Effect of C-Terminal Arginine Mutations on the Modulating Effect of Ethanesulfonate. If our hypothesis concerning the modulating effects of alkylsulfonates is correct, the effect should be abolished in the mutants that impair 2-KPC reduction but not 2-butanone reduction, i.e., R152A and R196A. To investigate this, the effect of ethanesulfonate on enantiomer yields was investigated for each of the C-terminal arginine mutants. These results are presented in Table 5. In the absence of ethanesulfonate, the percent yields of (*R*)- and (*S*)-2-butanol were all close to that of the wild-type enzyme except for R152A, where the percentage of (*S*)-2-butanol was increased somewhat. Of greater importance, the modulating effect of ethanesulfonate in increasing (*S*)-2-butanol yield was completely abolished in the mutants R152A and R196A, while the modulating effect was still observed for R203A and R209A (Table 5). These results provide further evidence that R152 and R196 are the key residues in binding the sulfonate of CoM and the key determinant in binding of the natural substrate to R-HPCDH.

Table 4: Effect of Alkylsulfonates on Kinetic Parameters for 2-Butanone Reduction by R-HPCDH[a]

modifier	K_m (mM)	change in K_m (*x*-fold)[b]	k_{cat} (s^{-1})	change in k_{cat} (*x*-fold)[b]	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
none	51.9 ± 0.5	1.0	0.027 ± 0.0009	1.0	0.52
sodium sulfate	52.7 ± 0.5	1.0	0.028 ± 0.001	1.0	0.53
methanesulfonate	17.2 ± 2.7	0.53	0.097 ± 0.004	3.5	5.6
ethanesulfonate	19.8 ± 3.3	0.61	0.11 ± 0.005	4.0	5.6
propanesulfonate	44.5 ± 2.2	1.4	0.040 ± 0.0007	1.5	0.90
butanesulfonate	88.0 ± 12.1	2.7	0.022 ± 0.001	0.82	0.25

[a] All assays were performed in triplicate at 30 °C with a fixed concentration of NADH (160 μM). Apparent kinetic constants were determined by fitting the data presented in Figure 3 to the standard form of the Michaelis−Menten equation. All effectors were present at 1 mM concentration. [b] Change in K_m or k_{cat} (*x*-fold) relative to assays with no effector present.

Scheme 3

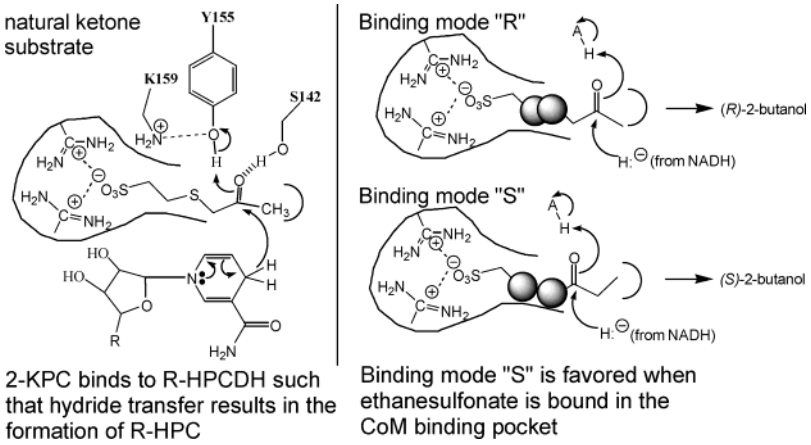


Table 5: Effect of C-Terminal Arginine Mutations on the Ability of Ethanesulfonate To Serve as an Enantioselective Modulator of 2-Butanone Reduction[a]

	no additions		+1 mM ethanesulfonate	
enzyme	% (*S*)-2-butanol	% (*R*)-2-butanol	% (*S*)-2-butanol	% (*R*)-2-butanol
WT	69.8 ± 0.9	30.2 ± 0.9	95.4 ± 0.3	4.6 ± 0.3
R152A	78.7 ± 0.3	21.3 ± 0.3	79.3 ± 0.1	20.7 ± 0.1
R196A	68.2 ± 0.2	31.8 ± 0.2	68.0 ± 0.3	32.0 ± 0.3
R203A	69.0 ± 1	31 ± 1	91.0 ± 0.2	9.0 ± 0.2
R209A	71.9 ± 0.2	28.1 ± 0.2	91.4 ± 0.1	8.1 ± 0.1

[a] All assays were performed in triplicate at 30 °C using 0.64 mg of R-HPCDH, NADH (15 mM), and 2-butanone (56 mM). Chiral gas chromatographic analysis of product ratios was determined as described in the Experimental Procedures. Percent yields are reported as means ± standard deviations.

DISCUSSION

The Sulfonate of CoM Is a Key "Substrate Component" Contributing to the Enantioselectivity of R-HPCDH. Among the SDR family of alcohol dehydrogenases, R- and S-HPCDH are distinguished by their unique ability to discriminate and produce enantiomers of short-chain alcohols. The present work provides compelling data suggesting that, in R-HPCDH, this discrimination results from the unique ability of the enzyme to recognize and bind the sulfonate moiety of the CoM functional group in an orientation leading to productive catalysis when the correct enantiomer of HPC is present. It is worth summarizing three pieces of evidence supporting the key role of the sulfonate. First, the enzyme exhibits identical K_m values but 600-fold different k_{cat} values for the enantiomers of R-HPC in the forward (oxidative) direction (Scheme 1). This demonstrates that either enantiomer will bind with the same high affinity but that only the *R*-enantiomer is oriented properly for catalysis (Scheme 2). Second, for the reverse (reductive) direction, R-HPCDH has a K_m for 2-KPC that is essentially identical to that for R-HPC in the forward direction (92 vs 100 μM, respectively). This result demonstrates that the oxidation state (carbonyl vs alcohol) and geometry (sp^2 trigonal planar vs sp^3 tetrahedral) of the substrate are of little importance in the overall affinity of substrate for R-HPCDH. Third, linear short-chain alkylsulfonates modulate the stereochemical outcome of 2-butanone reduction, in a saturable fashion and with K_{mod} values comparable to the K_m values for the natural substrates (K_{mod} values of 157 and 209 μM for ethanesulfonate and propanesulfonate, respectively). Together, these results support a model in which the sulfonate of CoM is integral to the binding of substrate and hence the enantioselectivity of R-HPCDH.

The length of the alkyl chain of the alcohol is also quite important in substrate affinity, as illustrated by the data presented in Figure 1 and Table 1. The K_m for aliphatic alcohols decreases by 1700-fold upon going from C3 2-propanol to C8 (*R*)-2-octanol. At that point, K_m is only 5-fold higher than for the natural substrate, but k_{cat} is still 15-fold less. These results are consistent with the idea that alkylsulfonate binding induces a conformational change in the enzyme that lowers the activation energy barrier for catalysis, leading to higher rates of catalysis. A similar effect was seen for the enantioselective effectors methanesulfonate and ethanesulfonate, which increased k_{cat} for 2-butanone reduction while at the same time lowering K_m (Figure 3 and Table 4).

With regard to the alkyl group, it is noteworthy that (*S*)-2-alkanols cease to be substrates for R-HPCDH when the chain length exceeds five carbon atoms. This observation, and the general trend for K_m values observed for the (*R*)- and (*S*)-2-alkanols (Table 1), is consistent with the model presented in Scheme 2. In order for an (*S*)-2-alkanol to be oxidized, the longer chain alkyl group (relative to the alcoholic carbon) will have to bind in the methyl binding pocket, while the methyl binding group will bind in the CoM pocket. This appears not to be a problem for (*S*)-2-butanol, where the K_m is equivalent to that for (*R*)-2-butanol. This result is consistent with previous observations that enantiomers of 1,2-epoxybutane are effective substrates for the four collective enzymes of epoxide carboxylation (*17*). Nucleophilic attack of CoM on (*R*)-1,2-epoxybutane would produce (*R*)-2-hydroxybutyl-CoM, which differs from R-HPC by the presence of an ethyl group rather than a methyl group on the alkyl side of the secondary alcohol. Thus, what we are referring to as a "methyl binding pocket" can apparently accommodate longer chain alkyl groups, including the ethyl and propyl groups. However, as the alkyl chain length becomes longer, it apparently can no longer be accommodated in this pocket due to steric hindrances, and hence the longer chain (*S*)-2-alkanols cannot bind properly and be oxidized by R-HPCDH. These observations and results are consistent with our observations that *X. autotrophicus* grows well with propylene, 1-butylene, and 1-pentene as carbon sources but poorly with longer chain alkenes (data not shown).

At this juncture it is worth commenting on the properties of CoM that make it so well suited as the cofactor for chiral alchol oxidation in the pathway of aliphatic alkene metabolism. CoM is the simplest organic cofactor known, consisting only of sulfonate and thiol functional groups separated by an ethyl linker (*18*, *19*). CoM is also one of the most specialized organic cofactors: its only known functions are as a methyl group carrier in Archaeal methanogenesis (*20*−*22*) and as the carrier of intermediates formed during aliphatic epoxide metabolism in alkene-oxidizing bacteria (*8*, *12*, *16*, *23*). While the thiol of CoA could arguably accomplish the same chemistry as CoM, the "nonbusiness end" of CoA is much larger and bulkier, a possible complication when a small, reactive thiol cofactor is needed for binding a small molecule (methyl group) in a constrained active site or allowing the discrimination of small enantiomers.

Interactions with Arginine Residues Are a Common Feature of CoM-Dependent Enzymes. Three-dimensional structures have been solved for two CoM-dependent enzymes: methyl-CoM reductase (MCR), the terminal methane-producing enzyme of methanogenesis (*24*), and 2-ketopropyl-CoM carboxylase/oxidoreductase (2-KPCC) (*25*), the enzyme that reductively cleaves 2-KPC, releasing free CoM and producing the reactive intermediate enolacetone, which is immediately carboxylated to acetoacetate (*13*). Importantly, the structures of both enzymes have been determined in the presence of substrate(s) and/or product. In the case of MCR, the structure of the enzyme with CoM and the second substrate CoB bound revealed that CoM was anchored to the polypeptide chain by a salt bridge between a specific arginine residue on a γ subunit and the sulfonate of CoM

(*24*). The structure of a complex of 2-KPCC with the product 2-KPC revealed that two arginine residues (R56 and R365) form salt bridges with the sulfonate of CoM (*25*). The presence of 2-KPC resulted in dramatic conformational changes in 2-KPCC relative to the substrate-free enzyme (*25*). It is likely that the strong salt bridges formed between the sulfonate of CoM and the two arginines are crucial to this conformational change.

The results discussed in this paper provide kinetic and biochemical evidence that arginine residues of R-HPCDH are also crucial to the recognition and binding of the sulfonate of CoM (or analogues), specifically, R152 and R196. The proposed salt bridges formed by these interactions (see Schemes 1−3) may also induce conformational changes in the active site, analogous to those reported for 2-KPCC (*25*), that orient residues within the active site for optimal catalysis. Note that the loss of *either* R152 or R196 dramatically lowered k_{cat} and raised K_m for the natural substrate 2-KPC (Table 2), as well as abolishing the "enantioselective modulator effect". On the basis of these results, it is possible that the sulfonate of CoM bridges R152 and R196 and that the resulting bridge is what introduces the conformational change, by pulling both arginine side chains in toward the sulfonate simultaneously (Scheme 3, left side). This idea is, of course, speculative, and confirmation of the roles these residues play in binding CoM awaits the determination of the three-dimensional structure of R-HPCDH. Toward this end, R-HPCDH has been crystallized (*26*), and the structure is currently being solved. In any event, we are confident in stating that arginine−sulfonate interactions are a key feature of the specialized function of CoM in this enzyme as others. No other known cofactor is capable of providing such an interaction.

Exploitation of the Uniqueness of CoM: A New Method for Controlling the Stereochemical Outcome of Ketone Reduction Using Enantioselective Effectors. The modulation of the stereochemical outcome of 2-butanone reduction by alkylsulfonates provides a new method for altering enzyme enantioselectivity. This modulating effect is in some ways analogous to chemical rescue of site-directed mutants of enzymes by the addition of molecules that mimic lost side chain functionality, e.g., addition of imidazole to histidine mutants. In the present case, the lost functionality (stereoselectivity) controlled by a portion of the substrate, rather than the enzyme, is restored by a separate molecule. It would be interesting to investigate whether the approach described here for modulating dehydrogenase stereoselectivity could be applied to a broader range of enzymes catalyzing a wider range of stereospecific transformations.

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