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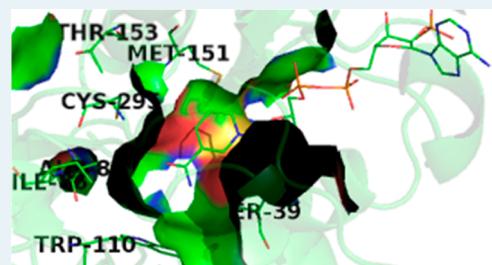
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ABSTRACT: The ability to control the substrate specificity and stereochemistry of enzymatic reactions is of increasing interest in biocatalysis. As this review highlights, this control can be achieved through various means, including mutagenesis of active site residues and alteration of physical variables such as temperature and pressure as well as through changing the reaction medium. Although the focus of this article is on alcohol dehydrogenase reactions, each of these techniques can be readily applied toward other enzyme classes, as well.



KEYWORDS: *mutagenesis, substrate specificity, alcohol dehydrogenase, stereospecificity, protein engineering, medium engineering*

1. INTRODUCTION

In recent years, biocatalysis, which is the use of enzymes as catalysts, has gained significant interest because biocatalysis can be seen as a means of “green chemistry.” For a process to be classified as green, it needs to generate or utilize few, if not zero, hazardous chemicals.¹ One very appealing aspect of enzymes is that they have been designed in nature for specific tasks, including recycling products and byproducts into other more useful compounds. Even when an enzyme’s active site has been optimized for a specific compound, the enzyme may show substrate promiscuity and thus accept a wide range of substrates *in vitro*. A wild-type enzyme can be modified by random or selective mutagenesis to expand the scope of the substrate specificity, some examples of which will be highlighted in this review. Two basic techniques of mutating an enzyme are through rational design and directed evolution.¹ Rational design entails altering a specific amino acid to cause a change in the enzyme selectivity, whether it is in the active site, the coenzyme binding pocket, or perhaps a spot elsewhere on the enzyme. Directed evolution involves developing a library through random mutagenesis and then selecting the mutants that have features that meet particular criteria, whether that be increased reactivity or decreased reactivity toward a desired substrate.

Another approach that is used to alter enzyme selectivity is changing the reaction medium (*i.e.*, medium engineering). This approach is of great interest because it represents an alternative approach to the more laborious protein engineering. In addition to protein engineering, this review will also cover a few examples of influencing stereoselectivity by switching reaction media from the traditional aqueous to nonaqueous media. The effect of temperature and pressure on stereospecificity of ADHs will also be described.

2. ALCOHOL DEHYDROGENASES

Alcohol dehydrogenases (ADHs, EC 1.1.1.X, X = 1 or 2) are nicotinamide adenine dinucleotide (NAD^+)- or nicotinamide adenine dinucleotide phosphate (NADP^+)-dependent oxidoreductases, which are enzymes that catalyze the transfer of electrons from a donor molecule to an acceptor molecule. They catalyze the reversible reduction of ketones and aldehydes to their corresponding alcohols. In an ADH-catalyzed redox transformation, a hydrogen molecule will be either added to a carbonyl or removed from an alcohol, as a hydride plus a proton. As shown in Figure 1, the hydride from the coenzyme (NADH/NADPH) can attack the carbonyl of an unsymmetrical ketone substrate from either the *Re* face or *Si* face, producing the corresponding optically active secondary alcohol, with only a few cases of enzymes allowing the hydrogen to come in from either face (*i.e.*, not stereospecific).

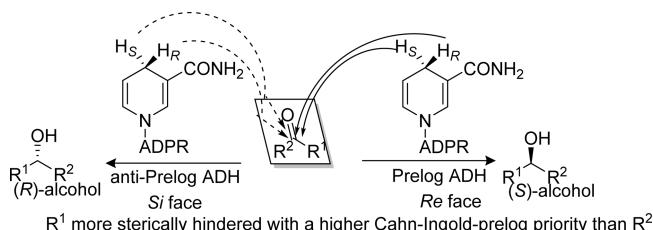


Figure 1. Prelog’s rule explaining the stereochemistry of the hydride transfer from NAD(P)H to the carbonyl carbon of a ketone substrate in ADH-catalyzed transformations. ADPR = adenosine diphosphoribose.

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To understand better the orientation of the substrate in the enzyme active site, it is important to have a map of the active site. In pioneering studies of the 1960s, Prelog proposed using a diamond lattice structure to visualize oxidoreductase active sites.² To validate his hypothesis, he tested the reaction with a series of increasingly larger substrates and was able to determine the maximum spatial limits of the active site in each direction. By overlaying the molecules which gave successful results, the active site was effectively mapped. It was important to have a means to map the active site because this would give a measure of the limit of stereospecificity and, in the future, substrate specificity.

In an extension of Prelog's diamond lattice model, Jones and co-workers further studied its utility toward horse liver alcohol dehydrogenase (HLADH) reactions.^{3–7} In the early 1980s, Jones and Jakovac proposed a cubic space section model because of the disadvantages of the diamond lattice for mapping an active site.³ Among the disadvantages were the use of sp³-hybridized carbon bond lengths and angles that could invariably limit researchers' use of the diamond lattice model to choose substrates for analysis. With the cubic space section model, the cube sizes were flexible, which allowed researchers to use blocks that were as large or small as desired. The results for simpler examples, which worked adequately with Prelog's diamond lattice, also worked satisfactorily with the cubic model. Jones and co-workers highlighted further examples that fit nicely with the new cubic shaped model.^{4–7}

Although mutagenesis can be accomplished routinely at present, it was not always the case. Hence, Jones studied the varying active sites of naturally occurring variants. Even though the cubic space model was useful, it was not widely adopted by other research groups because of the advent of more straightforward X-ray crystal structures, which give very clear information regarding the shape of the active site. Nonetheless, these early active site models have paved the way for current research because they clearly showed that substrate specificity is controlled by the shape of the active site, and hence, studying substrate specificity and then altering it is much more easily done with a model. For example, such a model has been proposed by Keinan et al.⁸ for a thermophilic alcohol dehydrogenase from *Thermoanaerobium brockii*, and the model includes a small and a large pocket, which is evident in the X-ray crystal structure.⁹ The main focus for this article will be on alcohol dehydrogenases of the medium chain family, but we will include a few other examples. Although there has been extensive research into enzyme mutation to alter cofactor specificity, that work falls outside of the scope of this review article, which will focus on altering substrate specificity through the use of mutagenesis, altering the temperature and pressure, and by changing the reaction medium.

3. EXAMPLES OF MUTAGENESIS TO ALTER SUBSTRATE SPECIFICITY

3.1. Medium Chain Alcohol Dehydrogenases. Site-directed mutagenesis has been widely utilized to study enzyme mechanisms and less commonly to alter substrate specificity. Oftentimes, a crystal structure has been used to guide the mutagenesis. Generally speaking, there are four reasons for altering protein structure through mutagenesis.¹⁰ Mutagenesis provides a means to study the rules that are in charge of protein structure and function. Mutation of an enzyme also can bring along a desired effect, which is one of the most common reasons. The last two reasons are the development of novel

strategies in designing and working with proteins and the opportunity to glimpse into the normal function of an enzyme.

3.1.1. Horse Liver ADH. HLADH has received a lot of attention over the years, in addition to having models applied to its active site as mentioned earlier in this review. In the 1970s, the isozymes of HLADH were discovered.¹¹ The ethanol and steroid monomer units, "E" and "S", could be combined in three different dimers, "EE," "ES," and "SS." The EE and SS isozymes differ in that only the SS form reacts with steroid alcohol. Interestingly, the E or S monomer units are inactive, but they become active when they dimerize. Whereas the SS isozyme reacts with ethanol as well as steroids, the EE isozyme reacts only with ethanol. Thus, the structural differences between the EE and SS isozymes that led to the changes in substrate specificity were of interest.

There were 10 differences in amino acid sequence found between the two forms of HLADH.¹² As shown in Table 1, the differences are scattered throughout the structure. These amino acid differences control the substrate specificity of the E and S isozymes of HLADH.

Table 1. Differences in Amino Acid Sequences of the Two Isozymes of HLADH

residue	E	S	AA ^a	location of residue in structure
17	E	Q	y ^b	surface
43	T	A		interior
59	T	A		surface
94	T	I	y	partly buried behind Phe-93 in the substrate pocket
101	R	S	y	cleft; minor subunit–subunit interaction
110	F	L	y	substrate pocket
115	D	Δ ^c	d	buried behind Leu-116 in the substrate pocket
172	I	V		interior
277	T	A		surface; close to the adenine ring of coenzyme
366	E	K	y	cleft between the two domains

^aDetermined by amino acid sequencing.¹³ ^b"y" indicates agreement between the cDNA-derived sequence and the protein sequence. ^cΔ, deletion. ^dResidue ambiguous in Jörnvall's determination.

After Plapp and co-workers highlighted the differences between the two isozymes, they were able to interconvert the E and S isozymes.¹⁴ Table 2 describes the mutations that were made for the E isozyme to react with a steroid alcohol. As Plapp found, simply deleting the Asp-115 introduced some steroid alcohol activity to the E isozyme. Surprisingly, the ESE mutant enzyme was found to have greater activity toward steroid alcohol than the S isozyme, even though the substrate binding pockets are identical. This implies that factors other than the amino acids in direct contact with the bound substrate could be in play.

Adolph et al. reported a ternary crystal structure of SS HLADH complexed with NAD⁺ and cholic acid and then compared the structure with that of the EE isozyme.¹⁵ Their study revealed that the major structural difference was the widening of the substrate channel.¹⁵ This allowed space for the steroid alcohol to fit in the substrate pocket, whereas the EE isozyme lacks the space for the steroid alcohol to fit in the pocket. These results emphasized the importance of the shape of the active site in substrate specificity of ADHs and demonstrated that there can be dramatic effects on substrate specificity with a relatively small number of mutations.

Table 2. Comparison of Primary Structures of the Natural and Mutant HLADH

residue no.	E	E/D115 Δ^a	ESE	ESS	S/K366E	S ^b
17	Glu	Glu	Glu	Glu	GLN	GLN
43	Thr	Thr	Thr	Thr	ALA	ALA
59	Thr	Thr	Thr	Thr	ALA	ALA
94	Thr	Thr	ILE	ILE	ILE	ILE
101	Arg	Arg	SER	SER	SER	SER
110	Phe	Phe	LEU	LEU	LEU	LEU
115	Asp	Δ^a	Δ^a	Δ^a	Δ^a	Δ^a
172	Ile	Ile	Ile	VAL	VAL	VAL
277	Thr	Thr	Thr	ALA	ALA	ALA
366	Glu	Glu	Glu	LYS	Glu	LYS
relative charge per monomer	-2	-1	-2	0	-1	+1

^a Δ , deletion. ^bAmino acids in capital letters correspond to S isozyme.

Table 3. Kinetic Parameters for Various Primary Alcohols with YADHs

ADH	alcohols; V/K _m (M ⁻¹ s ⁻¹)							
	ethyl	propyl	butyl	pentyl	hexyl	heptyl	octyl	nonyl
Sc I ^a	20 000	4 400	930	780	1 700	1 800	3 300	5 100
Sc T48S	12 000	2 500	1 300	330	480	ND ^b	ND	ND
Sc W57M	5 000	260	340	450	70	ND	ND	ND
Sc W93A	57	61	150	500	5100	26 000	29 000	13 000
Sc T48S/W93A	37	43	220	3 500	16 000	34 000	54 000	38 000
Sc T48S/W57M/W93A	530	140	160	90	90	460	820	930
HLADH	10 000	21 000	19 000	29 000	50 000	135 000	58 000	ND

^aSc: *Saccharomyces cerevisiae*. ^bND: not determined.

Table 4. Kinetic Parameters for Various Secondary and Branched Chain Alcohols with YADHs

ADH	V/K _m (M ⁻¹ s ⁻¹)								
	2-butanol			2-methyl-1-butanol					
	2-propanol	R	S	2-methyl-1-propanol	S	RS	3-methyl-1-butanol	benzyl alcohol	cyclohexanol
Sc I	25	0.8	18	7.6	NA ^a	NA	NA	NA	NA
Sc T48S	24	1.2	27	40	2.7	4.2	NA	14	NA
Sc W57M	12	1.4	3.3	2.5	NA	NA	NA	ND ^b	ND
Sc W93A	1.5	0.61	0.49	3.1	2.8	9.3	52	17	0.041
Sc T48S/W93A	ND	0.14	0.22	ND	16	57	230	5.0	1.1
Sc T48S/W57M/W93A	0.79	0.50	4.2	3.8	3.3	2.3	2.7	9.2	0.17
HLADH	24	110	290	13 000	18 000	10 000	19 000	78 000	5 500

^aNA, no measurable activity. ^bND, not determined.

3.1.2. Yeast ADH (YADH). YADH, an NAD⁺-dependent ADH, has in vivo activity toward acetaldehyde and ethanol during anaerobic fermentation of glucose. Just as with other ADHs, the active site of YADH accommodates various other aldehydes and their corresponding primary alcohols. Examples of unnatural substrates for YADH include acyclic aldehydes and their corresponding primary alcohols. Some of these unnatural substrates include 1-propanol and ethylene glycol, among many others.^{16,17}

In an effort to alter the substrate specificity of YADH, Murali et al. introduced the double mutant T48S/W93F YADH and found that this mutation opened up the active site as planned, thus allowing larger alcohols to fit inside the active site of the designed mutant.¹⁸ They also found that the Phe-93 position reduced the substrate affinity, possibly as a result of the hydrophobicity of the phenylalanine.

Three isozymes (I, II, and III) of YADH from *Saccharomyces cerevisiae*, and the ADH from *Schizosaccharomyces pombe* have nearly identical active sites.¹⁹ The only difference found was at

residue 294. Isozyme I and *S. pombe* ADH have a methionine at this position, whereas isozymes II and III have a leucine. Mutagenesis of the Met-294 in isozyme I allowed for comparison of the three isozymes, which yielded very similar kinetic parameters. The kinetic study was conducted with ethanol as the substrate, and all of the wild type enzymes and the mutant were tested. Isozyme II was found to have a 10–20 fold smaller Michaelis constant and inhibition constant than the others. This showed that an amino acid mutation outside the active site caused a significant change in enzyme activity, even though the active site sequences all matched.

In continuation of the previous work, Plapp and co-workers studied *S. cerevisiae* YADH I and compared it with the liver ADHs of horse and monkey.^{20,21} They also mutated residues within the active site of the YADH I. Table 3 shows the V/K_m values for these dehydrogenases with a range of primary alcohols containing 2–9 carbons. Interestingly, *S. cerevisiae* wild type YADH and its T48S mutant had decreasing activity from ethanol to pentanol, and then wild type activity increased with

Table 5. YADH: Substrate Specificity of YADH I and Its W54L Mutant with Various Alcohols^a

	wild type ADH I			mutant W54L		
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
Primary Alcohols						
ethanol	308 ± 11	4.0 ± 0.5 ^b	76.3 ± 7.1 ^b	102.1 ± 2.8	13.4 ± 0.6	7.64 ± 0.13
cinnamyl alcohol	133 ± 8	4.58 ± 0.35	29.0 ± 0.6	98.0 ± 3.9	1.5 ± 0.1	67.3 ± 2.9
Secondary Alcohols						
2-propanol	53.1 ± 10.1	268 ± 58	0.198 ± 0.005	5.8 ± 0.9	193 ± 33	0.030 ± 0.001
(S)-2-butanol	11.9 ± 3.5	93.3 ± 33.9	0.127 ± 0.008	0.31 ^b		

^aDetermined with 10 mM NAD⁺ in sodium pyrophosphate buffer (32 mM, pH 8.2), containing 100 mM Na₂SO₄ at varying alcohol concentrations at 25 °C. ^bWith an alcohol concentration of 50 mM.

Table 6. Kinetic Data for Wild-Type and Mutants of *Lactococcus lactis* ADH^a

aldehydes	LladhA (wild-type)			LladhA ^{RE1} (Y50F/I212T/L264V)			LladhA ^{29C8} (Y50F/N110S/I212T/L264V)		
	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
isobutyraldehyde	12	30	2.8	1.70	140	82	0.68	300	440
acetaldehyde	0.4	35	94	0.5	31	57	0.92	58	63
5-HMF ^b	22	19	0.88	0.67	23	34	0.57	29	51
2-furaldehyde	0.39	22	57	0.26	6.0	21	0.20	7	37
cinnamaldehyde	0.7	27	39	0.24	28	140	0.16	31	210

^aThe enzyme assays were conducted in 100 mM Tris–HCl buffer, pH 7, with 1 mM DTT, 200 μM NADH, and 10 mM substrate. ^b5-Hydroxymethyl-2-furfural.

the larger alcohols, and T48S YADH exhibited activity for hexanol and no activity beyond this. *S. cerevisiae* W57M YADH showed less ethanol specific activity, and the activity declined quickly as the primary alcohol was extended. Even though the single mutant *S. cerevisiae* W93A YADH had low activity for ethanol, the active site was enlarged enough that the larger primary alcohols reacted much more easily. Combining this mutation with the T48S mutant to generate the double mutant T48S/W93A YADH gave activity that was nearer to that of HLADH (see Table 3) with primary alcohols. However, the triple mutant (*S. cerevisiae* T48S/W57M/W93A) YADH had significantly lower activity toward most of the tested substrates. The secondary alcohol and branched chain alcohol specific activities shown in Table 4 demonstrate that the *S. cerevisiae* YADH mutants tested had little improvement with smaller alcohols but had some improvement with the larger secondary and branched alcohols. The activity was much lower than what was observed with HLADH.

Benner and co-workers tried to alter the substrate specificity of YADH to allow reactions with branched chain alcohols.²² There were a few residues that could have been potentially altered to increase the activity. The residues chosen (W54, L116, M270, I290) were pointed out by Branden et al. as side chains that could be modified to improve activity because they are located at the entrance of the active site.²³ Although HLADH has the same residues at locations 116 and 290, the methionine at site 270 for YADH is a valine in HLADH. At site 54, the tryptophan in YADH had been replaced by a leucine in HLADH. Benner investigated Trp-54 further to improve the activity of YADH.²² With the W54L mutation, the ethanol specificity dropped 10-fold compared with the wild type, as shown in Table 5. The greatest specificity for the W54L mutant was found with cinnamyl alcohol. Less change was found between the wild type and mutant enzymes for secondary alcohols exhibiting low activity. The W54L mutant showed improvement with longer straight chain alcohols and branched alcohols, which was very interesting.

3.1.3. *Lactococcus lactis* Alcohol Dehydrogenase (LladhA). LladhA is of interest because it can be used for the conversion of isobutyraldehyde to isobutyl alcohol, a possible biofuel, with NADH when expressed in *Escherichia coli*. Arnold and co-workers altered the active site of LladhA by site-saturation mutagenesis.²⁴ Even though the initial intent was to increase the specificity for isobutyraldehyde, the researchers also conducted kinetic assays with other aldehydes against LladhA, LladhA^{RE1} (Y50F/I212T/L264V), and LladhA^{29C8} (Y50F/N110S/I212T/L264V), with the results shown in Table 6.²⁴ As the wild-type LladhA was mutated to the triple and then quadruple mutant, the k_{cat} and k_{cat}/K_m for isobutyraldehyde went up considerably. Both acetaldehyde and 2-furaldehyde showed a decrease in k_{cat}/K_m , whereas k_{cat} for 5-hydroxymethylfurfural increased for both LladhA^{RE1} and LladhA^{29C8}. Interestingly, although the k_{cat} values for cinnamaldehyde showed little change, there was a 3–4 fold drop in the K_m values, which translated to a corresponding boost in the k_{cat}/K_m . In addition, the researchers found more substrates that worked for these three enzymes (wild type and two mutants), including cinnamaldehyde and 5-hydroxymethyl-2-furaldehyde.

3.1.4. *Sulfolobus solfataricus* ADH (SsADH). SsADH is a thermostable zinc-dependent ADH that has been isolated from *S. solfataricus*, obtained from a hot spring near Naples, Italy. Using error-prone PCR mutagenesis with screening for increased activity, Giordano et al. made the N249Y mutant SsADH, but unexpectedly, the mutation altered the substrate affinity, as well, as shown in Table 7.²⁵ The Asn-249 residue resides closer to the cofactor, as evidenced by the crystal structure shown in Figure 2. The substrate specificity decreased for most of the tested substrates, with a slight increase for 1-propanol. Interestingly, the values of both k_{cat} and K_m increased, resulting in net decreases in k_{cat}/K_m values for most substrates. The increase in k_{cat} was actually due to faster product release of the NADH cofactor in the steady state. Subsequently, Raia and co-workers did further studies with wild-type SsADH as well as trying out further mutations.²⁶ To determine if Trp-95 plays a role in the stereospecificity, they mutated the tryptophan to a

Table 7. Kinetic Constants for Wild-Type SsADH and N249Y SsADH (mSsADH)

substrate	k_{cat}/K_m (mM ⁻¹ s ⁻¹)	
	SsADH	N249Y SsADH
1-propanol	4.3	9.7
cyclohexanol	40	8.4
benzyl alcohol	13.8	14.5
4-methoxybenzyl alcohol	14.3	20.5
benzaldehyde	376.0	98.0
4-methoxybenzaldehyde	137.0	23.8

leucine, which has hydrophobic properties but less steric bulk. As shown in Table 8, the W95L mutation of SsADH reduced the specific activity with all of the alcohols tested, with some of the alcohols giving no measurable activity. Combining N249Y with the W95L mutation, the specific activity improved on each substrate tested but was still much lower than the wild-type SsADH. However, the *E* values for 2-butanol and 2-pentanol decreased from 42 and 7, respectively, for wild-type SsADH, and to 4 and 5 for W95L/N249Y mutant enzyme.

3.1.5. Thermoanaerobacter brockii ADH and Thermoanaerobacter ethanolicus secondary ADH (SADH). Two research groups independently isolated what were originally thought to be two different thermophilic bacteria, *Thermoanaerobacter ethanolicus* and *Thermoanaerobacter brockii*, from hot springs in Yellowstone National Park.^{27,28} When *T. ethanolicus* was isolated, it was targeted because a thermophile would allow anaerobic fermentations to be performed at temperatures near the boiling point of ethanol, theoretically increasing the efficiency of ethanol production. More recently, the two organisms were determined to be in the same genus, *Thermoanaerobacter*.²⁹ Bryant et al. isolated and characterized two alcohol dehydrogenases from *T. ethanolicus*.³⁰ It was observed that although one of the ADHs preferred primary alcohols, the other ADH was more active toward ketones and secondary alcohols than it was toward ethanol, and henceforth, this enzyme is referred to as secondary ADH (SADH). A similar SADH was reported by Keinan et al. from *T. brockii*.⁸ At first, the SADHs from the two organisms were thought to differ by four amino acids.³¹ However, they have been found recently to have identical sequences.³² The robustness of this thermophilic ADH has been of particular interest in designing

Table 8. Substrate Specificity for Wild-Type and Mutant SsADHs^a

substrate	k_{cat}/K_m (s ⁻¹ mM ⁻¹)		
	wild-type	W95L/N249Y	W95L
ethanol	0.67	0.06	<i>b</i>
1-propanol	10.4	2.4	<i>b</i>
1-butanol	38.7	9.4	<i>b</i>
(<i>S</i>)-2-butanol	100.0	0.16	<i>b</i>
(<i>R</i>)-2-butanol	2.4	0.044	<i>b</i>
1-pentanol	92.6	8.5	2.0
(<i>S</i>)-2-pentanol	37.1	0.76	<i>b</i>
(<i>R</i>)-2-pentanol	5.0	0.16	<i>b</i>
1-hexanol	57.1	15.1	10.5
1-heptanol	42.1	17.7	2.4
2-ethoxyethanol	6.4	<i>b</i>	<i>b</i>
3-pentanol	5.5	<i>b</i>	<i>b</i>
cyclohexanol	40.0	2.7	<i>b</i>
benzyl alcohol	13.8	5.2	2.9
4-methoxybenzyl alcohol	41.4	12.9	3.1
butyraldehyde	281.4	72.9	2.77
isobutyraldehyde	111.5	23.8	2.50
benzaldehyde	376.6	46.7	5.14
<i>trans</i> -cinnamaldehyde	233.3	82.1	2.30

^aThe activity was measured at 65 °C as described.²³ ^bNo measurable activity.

mutants without affecting the protein folding or thermal stability.

The crystal structure of wild-type SADH was published in 1998 by Frolov and co-workers under the PDB code 1YKF.⁹ Figure 3 displays the active site from the crystal structure in stereoview. The active site of SADH has a large pocket and small pocket, as predicted in Prelog's model and later by Keinan et al.⁸ Depending on how the ketone is oriented within the active site, the product alcohol would either be of *R* or *S* configuration. With this in mind, we mutated the Ser-39 into a threonine to modify the substrate specificity of SADH.³³ This mutation introduces a methyl group into the large pocket, decreasing the size of the large pocket and, hence, was predicted to favor *R* alcohols. The S39T mutation was selected because it did not disrupt the hydrogen bonding network in SADH, which is essential for the enzyme activity. As predicted,

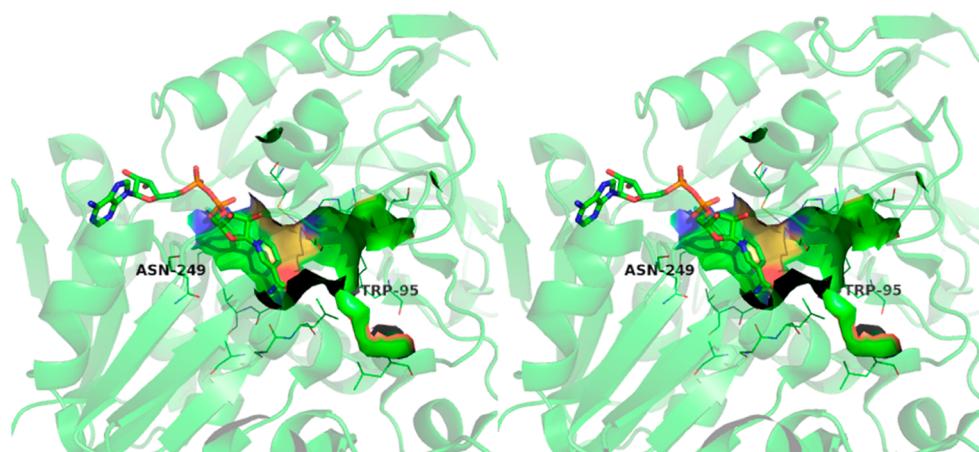


Figure 2. Crossed-eye stereoview of SsADH, along with two residues of interest labeled. NAD⁺ shown in stick-form. This image was prepared with PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC) using the PDB file (1R37).

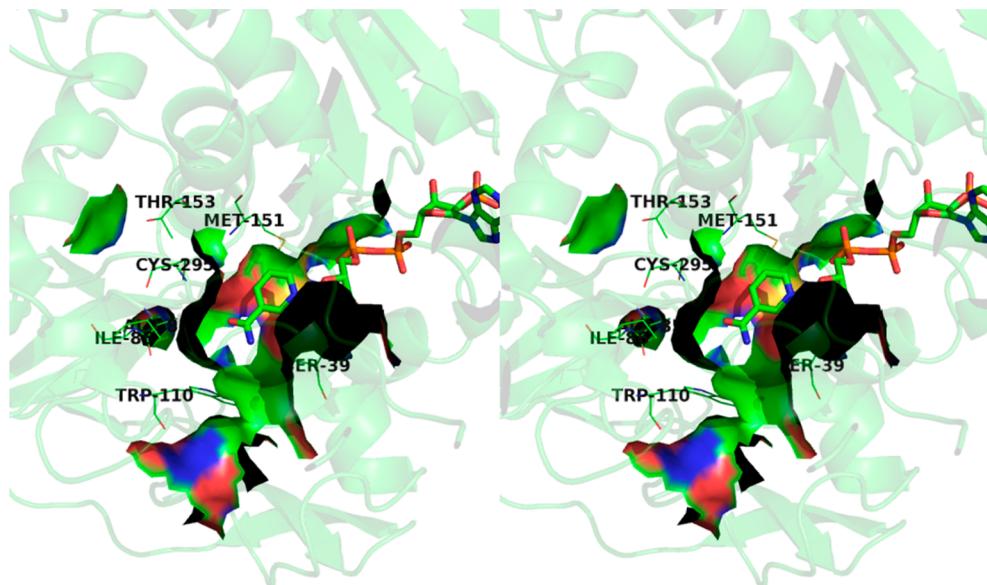


Figure 3. Crossed-eye stereoview of SADH, with residues of interest labeled. NADP⁺ shown in stick form. This image was prepared with PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC) using the PDB file (1YKF).

the stereospecificity for *R*-2-butanol and *R*-2-pentanol was increased significantly in S39T SADH. The relative specificity of wild-type and S39T mutant enzymes for secondary and primary alcohols was also of interest. The k_{cat}/K_m values for ethanol, 1-propanol, and 2-propanol decreased, with ethanol activity decreasing by 5-fold from wild-type to S39T SADH (Table 9). There was a 370-fold specificity ratio between 2-

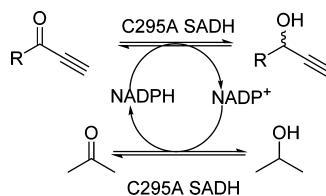
Table 9. k_{cat}/K_m Values for Oxidation of Ethanol, 1-Propanol, And 2-Propanol at 50 °C by Wild-Type and S39T SADH

substrate	k_{cat}/K_m ($M^{-1} s^{-1}$)	
	wild-type	S39T
ethanol	222 ± 36	45 ± 5
1-propanol	273 ± 35	206 ± 25
2-propanol	$(1.0 \times 10^5) \pm (2 \times 10^4)$	$(3.0 \times 10^4) \pm (0.7 \times 10^4)$

propanol relative to 1-propanol for wild-type SADH, whereas S39T had only a 145-fold specificity ratio of 2-propanol to 1-propanol.

We designed the C295A SADH mutant in an unsuccessful effort to eliminate the irreversible inactivation that occurred during asymmetric reduction of ethynyl ketones by wild-type SADH, which was thought to be due to the irreversible nucleophilic attack of the sulfur in Cys-295 on the ethynyl moiety of the substrates.³⁴ The C295A mutation introduced a nonnucleophilic residue in the small pocket, which improved the reaction but did not eliminate the inactivation noticed in wild-type SADH. Thus, the inactivation by ethynyl ketones may involve the reaction of Cys-37, which is an active site Zn ligand and, hence, cannot be mutated. However, many of the substrates gave better results with C295A SADH than with the wild type enzyme, and some substrates that show no activity with wild-type SADH were substrates for the mutant, as shown in Table 10.³⁵ The C295A mutation opened up the small pocket, thus allowing this mutant enzyme to accommodate butyl and substituted butyl moieties within the small pocket that are not accommodated by the small pocket of wild-

Table 10. C295A SADH-Catalyzed Reductions of Ethynyl Ketones^a



R	yield (%) ^b	abs. config. ^c	ee (%)
CH ₂ CH ₃	39 (32)	S (S)	76 (80)
CH(CH ₃) ₂	88 (50)	S (S)	>98 (>98)
C(CH ₃) ₃	39 (d)	S (S)	85 (85)
CH ₂ CH ₂ CH ₃	51 (28)	S (S)	76 (51)
CH ₂ CH(CH ₃) ₂	42 (20)	S (R)	56 (50)
CH ₂ C(CH ₃) ₃	0 (d)	– (R)	– (66)
CH ₂ CH ₂ CH ₂ CH ₃	60 (32)	S (R)	67 (42)
CH(CH ₃)CH ₂ CH ₂ CH ₃	43 (0)	S (–)	>98 (–)
CH ₂ CH ₂ CO ₂ CH ₃	23 (35)	R (R)	60 (82)

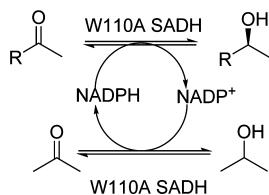
^aResults of the reductions with wild-type SADH are provided in parentheses. ^bYield reported as isolated yield. ^cAbs. config. = absolute configuration. ^dYield too low to isolate. ee determined by GC.

type SADH. Thus, the butyl and isobutyl ethynyl ketones in Table 10 gave products with stereochemical configuration opposite to that of the wild-type and C295A SADH. Furthermore, ketones with a large substituent containing an α -branch, such as *sec*-pentyl in Table 10, are good substrates for C295A SADH but are inactive with wild-type enzyme. It should be noted that the ethynyl group, which is smaller than the other substituent of the ketone, has higher Cahn–Ingold–Prelog priority for stereochemical assignment than alkyl groups, which leads to inversion of the stereochemical assignment (i.e., *S*-configured alcohols represent *anti*-Prelog products). Asymmetric reduction of methyl-4-oxohex-5-ynoate using C295A SADH resulted in the formation of the corresponding *R* alcohol with a decrease in enantioselectivity when compared with reduction using wild-type SADH, as shown in Table 10. The higher affinity of the small pocket of the active site of SADH

toward alkyl groups was first explained by Keinan et al. for SADH.⁸ When the ketone has only two small substituents, as methyl, ethyl, propyl, or isopropyl, the larger substituent paradoxically prefers to bind in the small pocket, probably because of stronger van der Waals interactions of the larger substituent in the small pocket.

To broaden the substrate specificity of SADH, we next designed W110A SADH, which enlarged the large pocket of the active site.³⁶ The W110A mutation allows for the reaction of phenyl-ring-containing ketone substrates and their corresponding *S*-configured alcohols that are not substrates for wild-type SADH. The stereochemistry of W110A SADH-catalyzed redox reactions follows Prelog's rule, in which NADPH delivers the pro-*R* hydride from the *Re* face of the prochiral ketone (Table 11). Surprisingly, phenylacetone was reduced with low

Table 11. Asymmetric Reduction of Phenyl Ring-Containing Ketones Using W110A SADH



R	Abs. Conf. of Product	Conv. (%)	ee (%)
PhCH ₂ CH ₂	<i>S</i>	99	>99
Ph(C=O)CH ₂	<i>S</i>	98	>99
(<i>E</i>)-Ph-HC=CH	<i>S</i>	64	>99
<i>p</i> -MeOC ₆ H ₄ (CH ₂) ₂	<i>S</i>	87	91
PhOCH ₂	<i>S</i>	>99	>99
<i>p</i> -ClC ₆ H ₄ CH ₂ CHCl	2 <i>S</i> ,3 <i>R</i>	83 ^a	>99
PhCH ₂	<i>S</i>	95	37
<i>p</i> -MeOC ₆ H ₄ CH ₂	<i>S</i>	97	>99
	<i>S</i>	>99	71

^aIsolated yield.

enantioselectivity, suggesting that phenylacetone can fit into two orientations within the active site of W110A SADH, allowing NADPH to deliver its hydride from either face of this substrate, therefore leading to selectivity mistakes.

The reduction of the α -chloroketone in Table 11 occurred with an unexpected dynamic kinetic resolution, probably because of the relatively high acidity of α -chloroketones. With the exception of β -tetralone, all reactions presented in Table 11 are reversible, which enabled the production of *R* alcohols via kinetic resolution by stereospecific oxidation of their racemates. The apparent irreversibility of β -tetralone reduction possibly

arises from the axial conformation of the substrate as initially formed in the active site, which then relaxes to the lower-energy equatorial conformation when it leaves the active site.

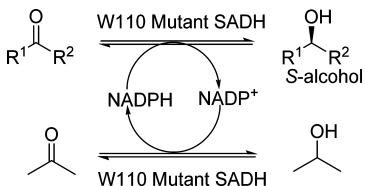
More recently, we used site-saturation mutagenesis of Trp-110 to study the effect of small changes in the large pocket of the active site of SADH on the stereoselectivity of aromatic ketone reductions.³⁷ Kinetic studies revealed that even a small alteration in the active site can make a significant change in stereoselectivity of ketone reduction. For example, there is a significant difference in kinetic parameters for W110V and those for W110G, as shown in Table 12. Five of the designed mutants (W110I, W110Q, W110M, W110V, W110L) reduced phenylacetone, 1-phenyl-2-butanone, and 4-phenyl-2-butanone with very high enantioselectivity (>99% ee), which represents a significant improvement when compared with reductions catalyzed by W110A and W110G mutants (Table 13). However, the W110M mutant did not show a higher *E* value than W110A, but showed a much lower value than the W110V, W110I, and W110L mutants (Table 12), so steric bulk is not the only factor affecting stereochemistry because the valine, isoleucine, leucine, and methionine side chains are similar in size.

To expand the small binding pocket of wild-type SADH, we mutated Ile-86 into alanine.³⁸ This mutant was designed to accommodate a phenyl ring within the small pocket because the wild-type small pocket can accommodate only a three-carbon substituent, and the mutation provides room for an additional three carbons. As predicted, I86A SADH shows high activity with acetophenone, which is not a substrate for the wild-type enzyme, and this mutant shows a reversal of stereochemistry, providing the *anti*-Prelog *R*-1-phenylethanol with >99% ee (Table 14). In contrast to other SADH mutants at Trp-110, which follow Prelog's rule, I86A SADH cannot accommodate the phenyl ring in the large pocket, and therefore, it follows *anti*-Prelog's rule in which NADPH delivers its pro-*R* hydride to the *Si* face of a prochiral ketone. Unfortunately, the largest substituent on the aromatic ring that the I86A mutant can accommodate is fluorine because any larger atom or group on the phenyl ring resulted in very low or no activity. Although the percent conversions in Table 14 provide estimates of the ease or difficulty of the substrates fitting into the active site, steric effects cannot be the only factor because the acetylpyridine isomers are sterically similar, but 3-acetylpyridine has lower conversion. Thus, electronic effects may also be involved.

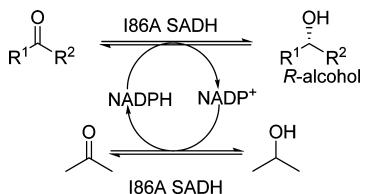
Recently, Reetz and co-workers utilized saturation mutagenesis using the above-mentioned work on SADH as a guide to identify mutation sites for asymmetric reduction of 4-alkylidene cyclohexanone prochiral ketones to their corresponding axially chiral alcohols.³⁹ The data shown in Table 15 highlight the extensive list of mutants used for this study. As noticed with the previously published studies of SADH,^{35–37} they found that, depending on the size of the substrate and opening made by the mutation in the active site, the stereoselectivity would shift in favor of one alcohol stereoisomer or the other. The *R* isomer was prevalent in the data from Table 15, with the conversions varying depending on the substrate. The best results were obtained with the Trp-110 mutants, which showed very high conversions and ee's. The lowest results were with the C295E mutant, which had very low conversions and moderate to high ee's. The most interesting results were with the Ile-86 mutants because they gave the *S* isomer for the smaller substituents and then eventually shifted to the *R* isomer as the substituent got larger. The conversions

Table 12. Kinetic Parameters for the Oxidation of (*S*)- and (*R*)-1-Phenyl-2-propanol with Mutant SADH

mutant SADH	enantiomer	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹)	$E = (k_{\text{cat}}/K_m)_S/(k_{\text{cat}}/K_m)_R$
W110I	<i>S</i>	18.8 ± 1.9	15200 ± 2300	80.3 ± 16.2
	<i>R</i>	0.46 ± 0.05	188 ± 25	
W110Q	<i>S</i>	2.3 ± 0.2	551.4 ± 31.2	80.0 ± 17.5
	<i>R</i>	0.025 ± 0.006	6.9 ± 1.5	
W110M	<i>S</i>	4.5 ± 0.4	1990 ± 230	16.3 ± 3.5
	<i>R</i>	0.045 ± 0.003	121.0 ± 22.1	
W110V	<i>S</i>	38.6 ± 3.06	45300 ± 4500	134.5 ± 27.7
	<i>R</i>	1.2 ± 0.3	336.5 ± 61.0	
W110L	<i>S</i>	0.65 ± 0.03	2510.0 ± 560	104.4 ± 42.1
	<i>R</i>	0.0080 ± 0.00081	24.0 ± 9.0	
W110G	<i>S</i>	17.8 ± 3.1	5800 ± 940	9.02 ± 2.6
	<i>R</i>	1.4 ± 0.3	639.0 ± 149.0	
W110A	<i>S</i>	31.1 ± 8.1	4935 ± 715	17.4 ± 4.7
	<i>R</i>	0.56 ± 0.09	284 ± 65	

Table 13. Asymmetric Reductions of Phenylacetone, 1-Phenyl-2-butanone, and 4-Phenyl-2-butanone by Mutant SADH

mutant SADH	phenylacetone		1-phenyl-2-butanone		4-phenyl-2-butanone	
	conv (%)	ee (%)	conv (%)	ee (%)	conv (%)	ee (%)
W110I	>99.9	>99.9	99.4	>99.9	99.1	>99.9
W110Q	>99.9	>99.9	83.5	>99.9	99.1	>99.9
W110M	>99.9	>99.9	97.3	>99.9	99.3	>99.9
W110V	>99.9	>99.9	99.2	>99.9	99.1	>99.9
W110L	>99.9	>99.9	98.9	>99.9	99.2	>99.9
W110G	>99.9	79	95.8	91.6	99.1	70.5
W110A	>99.9	84.1			99	>99.9

Table 14. Asymmetric Production of *anti*-Prelog Alcohols by the Use of I86A SADH

R ¹	R ²	conv (%)	ee (%)
C ₆ H ₅	CH ₃	79	98
C ₆ H ₅	CH ₃ CH ₂	60	>99
2,4-F ₂ C ₆ H ₃	CH ₃	33	>99
2-pyridyl	CH ₃	>99	>99
3-pyridyl	CH ₃	46	>99
4-pyridyl	CH ₃	>99	>99
3-thienyl	CH ₃	76	>99

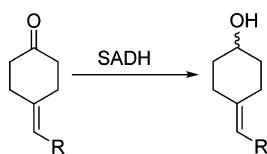
and ee's varied considerably, with the smaller groups giving better results, and the larger groups showing a much lower conversion and ee.

Although the SADH mutants mentioned so far in this section have given high enantiospecificity, there are uses that would

require a less specific enzyme. More specifically, for an enzyme to racemize an *R* or *S* alcohol, the enzyme in question would have to have an open enough active site to allow the substrate to bind in both orientations. An alcohol racemase, an enzyme that gives a racemic mixture starting from the corresponding enantiopure compound, would be of interest in coupling with a kinetic resolution method to achieve a dynamic kinetic resolution. It is of interest to alter ADHs to play the role of racemases because nature has very limited need for racemization, thus limiting the number of true racemases. The data in Table 16 show that *R* or *S* aromatic alcohols can be racemized by W110A SADH, at least partially.⁴⁰ This was made possible by facilitating the equilibrium in these redox reactions by including both NADP⁺ and NADPH in the reaction media.

3.1.6. *Rhodococcus ruber* ADH (ADH-A). The ADH from *Rhodococcus ruber* DSM 44541, known as ADH-A, catalyzes the asymmetric reduction of ketones to secondary alcohols. This zinc-dependent enzyme is structurally similar to HLADH.⁴¹ Gruber and co-workers published the crystal structure with an interest in the active site regions that would be ideal for mutagenesis to alter the substrate specificity.⁴¹ Figure 4 shows a 2-octanone molecule docked into the active site of ADH-A. From the illustration, one possible mutation would be of the Trp-295 into either an alanine or glycine, which would open up space in the active site for a larger substituent than methyl on 2-octanone. With this in mind, Widersten and co-workers recently published kinetic data for wild-type ADH-A and included some data on the H39N mutant, which had several-fold lower substrate specificity (k_{cat}/K_m) than the wild type enzyme for acetophenone and (*S*)-1-phenylethanol.⁴² They pointed out that the H39N mutation had no effect on the K_m of NAD⁺, meaning that the mutation substitutes well for the His-39 while altering the substrate specificity. It will be interesting to see if future mutations can affect the substrate specificity of this enzyme.

3.1.7. *Candida parapsilosis* carbonyl reductase (CPCR2). The carbonyl reductase from *Candida parapsilosis*, a zinc-containing ADH, accommodates medium-chain alcohols. Jakoblinnert and co-workers performed mutagenesis to expand the active site and thus widen the substrate scope of the enzyme.⁴³ The crystal structure and computational data were used to select residues (Leu-55, Pro-92, Gly-118, Leu-119, Leu-262) for site-saturation mutagenesis. They tested the resulting mutant library against poor substrates for the wild-type enzyme and found an interesting mutation, L119M. With 2-

Table 15. Performance of the Best SADH Mutants Specifically Evolved As Catalysts in Asymmetric Reduction of 4-Alkylidene Cyclohexanone Prochiral Ketones

mutation	R group											
	bromo		methyl		phenyl		methyl ester		ethyl ester		isopropyl ester	
	conv (%)	ee (%)	conv (%)	ee (%)	conv (%)	ee (%)	conv (%)	ee (%)	conv (%)	ee (%)	conv (%)	ee (%)
none	≥95	66 (R)	92	91 (R)	38	77 (R)	26	89 (R)	33	87 (R)	42	92 (R)
A85V	≥95	95 (R)	90	91 (R)	28	63 (R)	18	84 (R)	22	91 (R)	26	93 (R)
I86A	≥95	98 (S)	≥99	74 (R)	33	65 (R)	13	75 (R)	18	85 (R)	29	95 (R)
I86G	≥95	98 (S)	≥99	82 (S)	30	66 (R)	24	24 (S)	25	8 (R)	24	93 (R)
I86E	≥95	95 (S)	≥99	84 (S)	22	88 (R)	18	58 (R)	30	89 (R)	31	92 (R)
I86M	≥95	92 (S)	≥99	25 (S)	27	73 (R)	35	84 (R)	52	86 (R)	46	90 (R)
I86T	≥95	92 (S)	89	61 (S)	15	74 (R)	21	40 (R)	23	57 (R)	27	93 (R)
W110A	≥99	82 (R)	≥99	93 (R)	≥99	99 (R)	≥99	98 (R)	≥99	99 (R)	≥99	99 (R)
W110E	≥99	91 (R)	≥99	92 (R)	≥99	89 (R)	≥99	91 (R)	≥99	92 (R)	≥99	91 (R)
W110M	≥99	97 (R)	≥99	95 (R)	≥99	99 (R)	≥99	98 (R)	≥99	99 (R)	≥99	99 (R)
W110T	≥99	97 (R)	≥99	92 (R)	≥99	97 (R)	≥99	98 (R)	≥99	99 (R)	≥99	99 (R)
C29SE	16	84 (R)	27	79 (R)	10	88 (R)	8	65 (R)	10	64 (R)	14	72 (R)

Table 16. W110A SADH-Catalyzed Racemization of Enantiopure Phenyl-Ring-Containing Secondary Alcohols

entry	R	substrate	E value (S/R)	ee (%)	
				before	after
1	PhCH ₂	S	3.4	>99	6.3 (S)
2	PhCH ₂	S	3.4	>99	4.3 (S) ^a
3	PhCH ₂	R	3.4	>99	10.3 (R)
4	PhCH ₂	R	3.4	>99	15.4 (R) ^a
5	p-MeOC ₆ H ₄ CH ₂	S	12	91	44.0 (S)
6	PhCH ₂ CH ₂	R	>100	>99	34.5 (R)
7	PhCH ₂ CH ₂	S	>100	99	82.4 (S)
8	PhCH ₂ CH ₂	S	>100	72	51.8 (S)
9	PhOCH ₂	S	40	>99	>99 (S)

^aDMF used instead of acetonitrile as the cosolvent.

methylcyclohexanone as the substrate, the specific activity increased by 7-fold with L119M, as compared with wild-type CPCR2. Other substrates of interest listed in Table 17 showed an increase in activity with L119M CPCR2 as compared with wild-type CPCR2 for acetophenone. With cyclohexanone and 4-methylcyclohexanone, the increase in activity for L119M CPCR2 was around 4-fold.

3.2. Other ADH Families. **3.2.1. *Sporobolomyces salmonicolor* Carbonyl Reductase (SsCR).** Through the application of substrate–enzyme docking-guided point mutations, Zhu and co-workers have developed a set of useful *Sporobolomyces salmonicolor* carbonyl reductase (SsCR) mutants with enantioselectivity that is different from the wild-type enzyme.⁴⁴ As Table 18 demonstrates, the R alcohols were the products from wild-type SsCR, whereas mutations at residue Gln-245 shifted the isomeric product to the S-alcohols. Li and co-workers subsequently found that mutation of Met-242 resulted in formation of S alcohols for many of the substrates tested, as shown in Table 19.⁴⁵ Interestingly, the best mutant depended

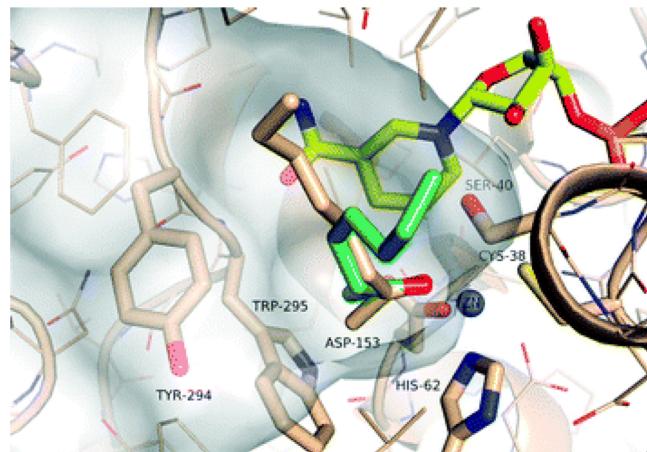
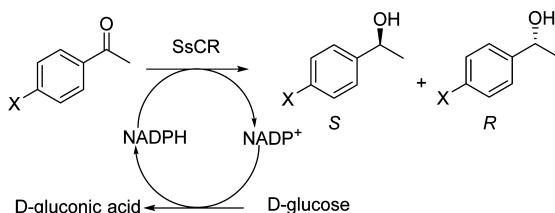


Figure 4. Results of docking 2-octanone into active site of ADH-“A” showing a comparison of representative binding modes from the two lowest energy docking clusters. The active site pocket is shown in a semitransparent surface representation. Amino acids and the docked 2-octanone are shown in pink and green, respectively, and the cofactor is in yellow. The zinc ion is shown as a gray sphere. The figure was prepared using PyMOL. (Figure and caption reprinted with permission of The Royal Society of Chemistry from ref 41).

Table 17. Specific Activities of wtCPCR2 and L119M-CPCR2^a

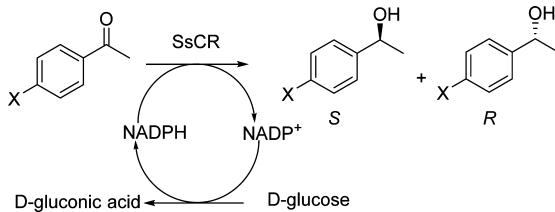
substrate	specific activity (U/mg)	
	Wt-CPCR2	L119M-CPCR2
acetophenone	26.5	16.2
2-methylcyclohexanone	3.0	8.5
3-methylcyclohexanone	16.8	37.7
4-methylcyclohexanone	18.3	95.6
cyclohexanone	35.8	144.6

^aActivities were determined at 5 mM substrate concentration employing the standard NADH-depletion assay; measurements were conducted in triplicate.

Table 18. Asymmetric Reductions of para-Substituted Acetophenones Catalyzed by SsCR and Its Q245 Mutants

X	WT-SsCR		Q245H-SsCR		Q245P-SsCR		Q245L-SsCR	
	specific activity ^a	ee (%)						
OCH ₃	20	57 (R)	16	79 (S)	62	98 (S)	20	96 (S)
H	28	42 (R)	85	78 (S)	39	64 (S)	86	82 (S)
F	14	46 (R)	72	92 (S)	25	90 (S)	36	93 (S)
Cl	20	14 (R)	238	90 (S)	309	96 (S)	67	96 (S)
Br	13	42 (R)	203	92 (S)	403	98 (S)	47	97 (S)
CH ₃	11	59 (R)	25	95 (S)	45	96 (S)	20	95 (S)
C(CH ₃) ₃	11	31 (R)	32	96 (S)	84	99 (S)	9	99 (S)

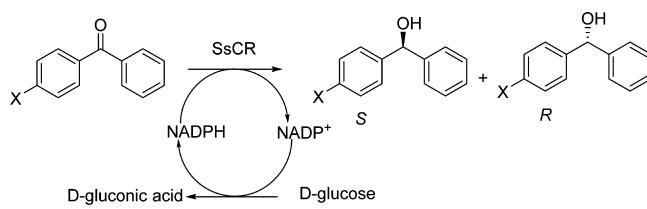
^aThe specific activity is defined as nmol/min·mg⁻¹

Table 19. Reduction of para-Substituted Acetophenones with M242/Q245-SsCR

substrate	enantiomeric excess (%)								
	WT-SsCR	M242Y	M242D	M242C	M242G	M242L/Q245P	M242F/Q245T	M242C/Q245L	M242L/Q245T
H	42 (R)	12 (S)	82 (S)	13 (S)	54 (S)	74 (S)	42 (S)	17 (S)	58 (S)
F	46 (R)	41 (S)	90 (S)	54 (S)	70 (S)	92 (S)	90 (S)	16 (S)	94 (S)
Cl	14 (R)	36 (S)	77 (S)	27 (S)	62 (S)	99 (S)	94 (S)	50 (S)	98 (S)
Br	42 (R)	22 (S)	61 (S)	4 (R)	52 (S)	>99 (S)	89 (S)	24 (S)	99 (S)
CH ₃	59 (R)	21 (R)	43 (S)	38 (R)	4 (S)	99 (S)	72 (S)	20 (S)	95 (S)
OCH ₃	57 (R)	7 (R)	39 (S)	18 (R)	6 (R)	99 (S)	92 (S)	36 (S)	97 (S)
C(CH ₃) ₃	31 (R)	93 (S)	>99 (S)	96 (S)	90 (S)	>99 (S)	95 (S)	99 (S)	99 (S)
CF ₃	17 (R)	28 (S)	37 (S)	5 (S)	17 (S)	>99 (S)	98 (S)	94 (S)	99 (S)

on the substrate being tested. Combining mutations at Met-242 and Gln-245 provided double-mutant enzymes that gave the S alcohols as the products, which also can be seen in Table 19. Expanding on the acetophenone study, Zhu and co-workers tested the Gln-245 mutants against benzophenones, including 4-methylbenzophenone and 4-chlorobenzophenone, as shown in Table 20.⁴⁶ The wild type SsCR gave the R alcohol in high ee for each of the two substrates, but the Q245L mutant SsCR gave R alcohols with lower ee. Interestingly, the Q245P mutation shifted the preference toward the S alcohol for both 4-methylbenzophenone and 4-chlorobenzophenone in moderate ee. The Q245H mutation retained the R alcohol preference for 4-methylbenzophenone in moderate ee, and 4-chlorobenzophenone gave the S alcohol in low ee.

3.2.2. *Candida tenuis* Xylose Reductase (CtXR). *Candida tenuis* xylose reductase (CtXR, EC 1.1.1.21),⁴⁷ a member of the NAD(P)-dependent aldo–keto reductase superfamily, was unexpectedly found to catalyze the asymmetric reduction of a series of aromatic α -keto esters to their corresponding optically active α -hydroxy esters, as shown in Table 21.⁴⁸ The produced α -hydroxy esters had the R configuration (Prelog's mode) with very high enantioselectivities (>99% ee). An energy-minimized

Table 20. Reduction of 4-Methylbenzophenone and 4-Chlorobenzophenone with SsCR Enzymes

enzyme	X = methyl		X = chloro	
	conv (%)	ee (%)	conv (%)	ee (%)
wild-type	>99	84 (R)	95	78 (R)
Q245P	99	48 (S)	98	76 (S)
Q245L	98	46 (R)	>99	28 (R)
Q245H	94	46 (R)	>99	22 (S)

docking study revealed steric conflicts between the indole ring of Trp-23 and carbonyl group substituents, as shown in Figure 5. Replacement of Trp-23 by smaller amino acids such as Phe or Tyr using site-directed mutagenesis resulted in up to 8-fold enhancement in catalytic efficiency for aromatic α -keto esters,

Table 21. Steady-State Kinetic Analysis of Reduction of Aromatic α -Keto Esters Catalyzed by Wild-Type CtXR and W23F and W23Y Mutants

substrate	$k_{\text{cat}}/K_m (\text{M}^{-1} \text{s}^{-1})$			
	wild-type CtXR	W23F CtXR	W23Y CtXR	
D-xylose	136	2.4	0.8	
X = H, Y = H, Z = H	269	911	636	
X = Cl, Y = H, Z = H	576	4 029	3 158	
X = H, Y = Cl, Z = H	53	369	186	
X = H, Y = H, Z = Cl	2 211	13 835	11 056	
X = CN, Y = H, Z = H	27	208	128	
X = H, Y = CN, Z = H	21	131	74	
X = H, Y = H, Z = CN	197	955	717	

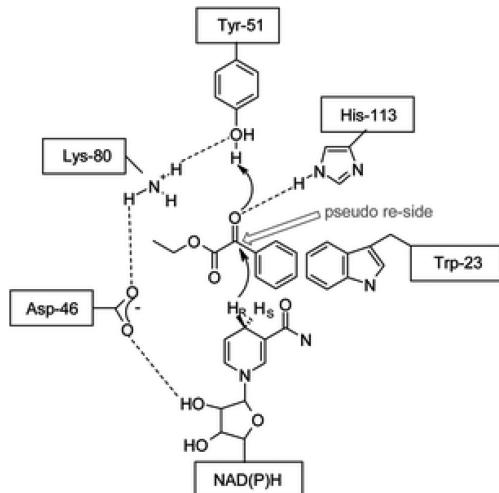


Figure 5. Proposed orientation of an aromatic α -keto ester in the active site of CtXR and stereochemical course of asymmetric production of α -hydroxy esters. Figure reprinted with permission of The Royal Society of Chemistry from *Chem. Commun.* 2007, 1047–1049.

as shown in Table 21. W23F and W23Y mutant variants of CtXR retain the high enantioselectivity of wild-type CtXR in asymmetric reduction of aromatic α -keto esters.

Nidetzky and co-workers also tested the wild type and aforementioned CtXR mutants against a series of ketones, which are provided in Table 22.⁴⁹ The introduction of a phenylalanine or tyrosine mutation at Trp-23 caused mixed effects with the chosen ketones, including the largest increase with oxopantoyl-lactone, giving a 4- to 5-fold specific activity increase compared with wild-type CtXR.

4. OTHER FACTORS AFFECTING ACTIVITY AND STEREOSELECTIVITY OF ADHS

4.1. Effects of Temperature, pH, and Pressure on Stereoselectivity. An important goal of research in biocatalysis is to exert a greater influence on the stereoselectivity of the enzymatic reaction. An obvious way to achieve it is by optimizing the physical conditions—that is, temperature, pressure, pH, etc.—of the given enzymatic reaction. However, for most enzymes, this is rather difficult to do, given that most enzymes are active only in a very narrow temperature

Table 22. Wild-Type and Mutant CtXR Kinetic Parameters with a Series of Ketones

substrate	$k_{\text{cat}}/K_m (\text{M}^{-1} \text{s}^{-1})$		
	wild-type CtXR	W23F CtXR	W23Y CtXR
acetophenone	0.5	0.2	0.2
acetoin	1.8	0.8	0.8
4-hydroxybutan-2-one	0.05	0.05	0.03
diacetyl	800	282	121
acetylacetone	0.07	0.03	0.03
oxopantoyl-lactone	8.0	46	36
ethyl pyruvate	542	160	57
ethyl benzoylformate	269	911	636
ethyl acetoacetate	0.7	0.3	0.2
ethyl 4-chloroacetoacetate	34	68	62
ethyl 4,4,4-trifluoroacetoacetate	0.3	0.6	0.3

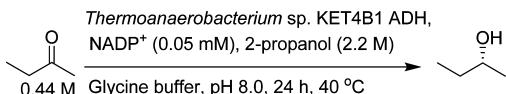
range, and stability often rapidly declines past 42 °C. However, there are enzymes from thermophilic bacteria that can be used to study the effects of temperature on stereoselectivity because these specific enzymes are stable over a much wider range of temperatures. One of the earliest such examples of ADHs was studied by Keinan et al., who reported the reduction of 2-pentanone to (*S*)-2-pentanol by SADH at temperatures as high as 50 °C, although the highest stereoselectivity was observed at 5 °C.⁸

We found a strong temperature dependence on enantiospecificity of SADH, and it was observed that for 2-butanol, there was a reversal of stereospecificity from *S* below 26 °C to *R* above that temperature. As Keinan had reported,⁸ (*S*)-2-pentanol was found to be the preferred substrate at temperatures up to 60 °C.^{50,51} The effect of temperature on enantiospecificity was fit to eq 1. The effect of temperature was found to be the result of a relatively large $\Delta\Delta S^\ddagger$, which favors the reaction of the *R* enantiomer because $T\Delta\Delta S^\ddagger$ increases with temperature. This was the earliest report that quantitatively established the influence of temperature in stereospecificity of SADH and showed great potential for its practical applications.

$$-RT \ln E = \Delta\Delta H^\ddagger - T\Delta\Delta S^\ddagger \quad (1)$$

Keinan et al. also studied the effect of pH on the reduction of 2-pentanone by SADH.⁸ They observed a correlation between the pH and the stereospecificity of the formed product, 2-pentanol, with the best enantiopurity obtained at pH 7.5–8. We studied the effect of pH on stereospecificity of SADH on 2-butanol, and we observed that the *E* value for 2-butanol increases from 2.5 at pH 9 to 4.2 at pH 5.5.⁵² The increase in stereospecificity was attributed to differences in catalytic commitments for the two enantiomers, which causes a change in the apparent kinetic pK_a of the enzyme for the two enantiomers. The catalytic commitment is the ratio of k_{cat} to the rate constant for release of the unreacted substrate from the enzyme. If this ratio, *C*, is >1 , the apparent kinetic pK_a for the reaction of the substrate is shifted to lower pH by $\log(1 + C)$. This represented yet another correlation of a physical parameter change to stereospecificity, and it potentially imparted one more tool in the hands of synthetic chemists to influence stereospecificity.

The next step was to study the cumulative effect of SADH mutations and physical parameters. We studied the effects of temperature on stereospecificity of 2-butanol and 2-pentanol oxidation using S39T SADH.³² An interesting effect of this

Table 23. Concentration Effect of Organic Cosolvents on Enantioselectivity of *Thermoanaerobacterium* sp. KET4B1

concn organic solvent (% v/v)	acetonitrile	methyl propionate	methanol	methyl formate	dimethyl sulfoxide	heptane	% ee ^a
0	23	23	23	23	23	23	23
10	34	49	27	55	62	24	
20	48	53	34	61	56	n.d. ^b	
40	65	64	49	n.d.	n.d.	n.d.	

^aPercent ee values were determined at ~60% conversion. ^b"n.d." indicates not determined due to high enzyme inhibition.

mutation was that it increased the preference for (*R*)-2-butanol and (*R*)-2-pentanol, and this represented a potential route for preparing both enantiomers of selected alcohols using the wild-type and S39T mutant SADH, respectively. The thermodynamic parameters, $\Delta\Delta H^\ddagger$ and $\Delta\Delta S^\ddagger$, were compared for wild-type and S39T enzymes with 2-butanol and 2-pentanol, and the change in stereochemistry was found to be associated with a change in the $\Delta\Delta H^\ddagger$. The effects of temperature on the reaction of C295A SADH with 2-butanol and 2-pentanol were also determined.⁵³ Surprisingly, the temperature dependence of the enantiospecificity of 2-butanol and 2-pentanol is much less for C295A than for wild-type SADH because of very small values of $\Delta\Delta S^\ddagger$. This is consistent with the $\Delta\Delta S^\ddagger$ arising from a difference in desolvation of the active site on substrate binding; the crystal structure of wild-type SADH shows a structural water molecule hydrogen bonded to Cys-295.

There have been only a few reports on the effect of high pressure on enzymatic reactions. This should not be surprising because temperature can be manipulated by common inexpensive instrumentation available in every laboratory, whereas application of high hydrostatic pressure requires expensive specialized instrumentation. Morita and Haught showed that malic dehydrogenase from *Bacillus stearothermophilus* was inactive at 101 °C from 0.1 to 70 MPa;⁵⁴ however, there was activity observed at 70 MPa and optimal activity at 130 MPa at 101 °C. This showed that pressure can increase enzyme activity, and Dallat and Legoy showed that SADH was activated by pressure up to 100 MPa.⁵⁵ Cho and Northrop also studied the kinetics of YADH under high pressure.⁵⁶ There are even fewer examples of hydrostatic pressure studies on enzyme stereochemistry. Kahlow et al. studied the effects of pressure on *Candida rugosa* lipase-catalyzed transesterification of menthol in chloroform at high pressure and found the *E* value to significantly decrease with pressure from an *E* of 55 at 0.1 MPa to ~9 at 10 MPa.⁵⁷ A model was developed on the basis of molecular dynamics, and it showed that changes in active site solvation were responsible for the observed effect.

Recently, we studied the effects of hydrostatic pressure and temperature on stereospecificity of S39T SADH.⁵⁸ It was observed that the S39T SADH was active under high pressure and temperature conditions, for example, 137.5 MPa and 325 K. The enantiomeric ratio, *E* value (*R/S*) was observed to be ~2 for 2-butanol and nearly 1 for 2-pentanol under different temperature and pressure conditions. A greater effect on pressure was observed for 2-hexanol, with the *S* isomer being the preferred enantiomer.

The *E* value for 2-hexanol varied from 0.25 at 298 K and atmospheric pressure to 0.08 at 137.5 MPa, which was a 3-fold decrease in the *R/S* ratio. The combined temperature and pressure data were fitted to eq 2 to obtain the $\Delta\Delta S^\ddagger$ and $\Delta\Delta V^\ddagger$ values of +46 ± 15 J/mol and +(2.0 ± 0.4) × 10⁻² L/mol, respectively. Thus, the effects of temperature and pressure are opposite, with temperature favoring the *R* enantiomer and pressure favoring the *S* enantiomer. These results support our previously proposed solvation model,⁵⁹ which postulates that the positive entropy difference results from the expulsion of a water molecule that occupies the small pocket of the SADH active site in the crystal structure, when a larger alkyl group of the *R* alcohol is bound in it. As further supporting evidence for this theory, the magnitude of $\Delta\Delta V^\ddagger$ from data fitting, 0.02 L/mol, is approximately the same as the volume of 1.0 mol of water.

4.2. Effect of Reaction Medium in Activity and Selectivity of ADHs. There are a number of advantages of using nonaqueous media instead of the natural aqueous medium in biotransformations.⁶⁰ There is no doubt that using enzymes in nonaqueous media expands the scope of their use in production of enantiopure compounds because many of the interesting substrates are either water-insoluble or sparingly soluble. The use of nonaqueous media in biotransformations also allows their use with other interesting reactions. Klibanov and co-workers reported the first example that shows that enzymes, including dehydrogenases, can function in nearly anhydrous nonaqueous media.⁶¹ Since then, the concept of "reaction medium engineering" has gained significant interest in the field of biocatalysis.⁶² A significant amount of research has been devoted to study the effect of reaction medium on enzyme activity and selectivity. Numerous examples of ADH-catalyzed transformations have been conducted in nonaqueous reaction media, including organic solvents, ionic liquids, and supercritical CO₂. In this review, we focus on examples that show the effect of reaction medium on stereoselectivity of ADH-catalyzed redox reactions.

Simpson and Cowan studied the effect of using organic cosolvents on enantioselectivity of asymmetric reduction of aliphatic ketones by secondary ADH from *Thermoanaerobacterium* sp. KET4B1.⁶³ They reported an enhancement in enantioselectivity of reduction reactions of 2-butanone to (*R*)-2-butanol when carried out in various organic solvents at different concentrations (Table 23). They noticed that solvents with lower log *P*, hydrophobicity constant, values have more significant influences on enantioselectivity; however, no direct correlation was observed. Surprisingly, the enantioselective reduction of 3-hexanone to (*S*)-3-hexanol in 10% (v/v) and 20% (v/v) of acetonitrile resulted in a small decrease in ee from

$$-RT \ln E = \Delta\Delta H^\ddagger - T\Delta\Delta S^\ddagger + P\Delta\Delta V^\ddagger \quad (2)$$

75% to 68% and 53%, respectively. Under the same conditions and using 10% (v/v) of acetonitrile, 3-methylbutan-2-one resulted in the formation of (*R*)-3-methylbutan-2-ol in 99.9% ee, in comparison with 98.5% ee in pure aqueous medium, which, as claimed by the authors, could be within the error of the GC that was used to determine optical purity.

Schumacher et al. studied the influence of water-miscible organic solvents on the enantioselective reduction of 2-butanone to (*R*)-2-butanol using the *anti*-Prelog *Lactobacillus brevis* ADH.⁶⁴ They noticed an enhancement in the enantioselectivity when acetonitrile or 1,4-dioxane are used as cosolvents. They studied the effect of varying the organic solvent concentration from a mole fraction of 0.015 to 0.1 mol with a minimum enantioselectivity of 33% ee at $X_{\text{acetonitrile}} = 0.015$ and a slight increase to 43% ee at $X_{\text{acetonitrile}} = 0.05$, as shown in Table 24. They also noticed a minimum enantioselectivity of

Table 24. Influence of Different Organic Solvent Content on Enantioselectivity of 2-Butanone Reduction by *Lactobacillus brevis* ADH

entry	solvent	X (mole fraction of organic solvent)	ee (%)
1	acetonitrile	0.015	33
2		0.025	41
3		0.050	43
4		0.100	43
5	1,4-dioxane	0.015	34
6		0.025	32
7		0.050	34
8		0.100	40
9	phosphate buffer		37

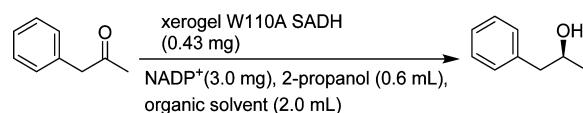
32% ee at $X_{1,4\text{-dioxane}} = 0.025$ and an enhancement to 40% ee at $X_{1,4\text{-dioxane}} = 0.1$. It is worth mentioning that the same enantioselective reduction reaction gave (*R*)-2-butanol in 37% ee when carried out in phosphate buffer solution.

The high tolerance of SADH to elevated concentrations of organic solvents enabled us to use xerogel-encapsulated W110A SADH in hydrophobic organic solvents using 2-propanol as a cosubstrate.⁶⁵ A significant enhancement in ee of the enantioselective reduction of phenylacetone is noticed when the reaction is carried out using xerogel-encapsulated W110A SADH in organic solvents in comparison with conducting the same reaction using free enzyme in aqueous media, as shown in Table 25.

Eckstein et al. reported an ADH-catalyzed reduction reaction in a biphasic system containing the ionic liquid [BMIM][NTf₂] as the nonaqueous phase.⁶⁶ They noticed that the *L. brevis* ADH-catalyzed *anti*-Prelog enantioselective reduction of 2-octanone is faster in a biphasic system containing [BMIM]-[NTf₂] than when the same reaction is conducted using methyl *tert*-butyl ether as the nonaqueous solvent. They claimed that this improvement was due to the favorable partition coefficients of the cosubstrate 2-propanol and coproduct acetone, with the latter preferring to reside in the [BMIM][NTf₂] layer, which shifts the equilibrium toward the reduction pathway.

Driven by the high tolerance of SADH to elevated concentrations of nonaqueous solvents, we investigated the effect of various organic solvents and ionic liquids on

Table 25. Enantioselective Reduction of Phenylacetone in Organic Solvents Using Xerogel-Encapsulated W110A SADH

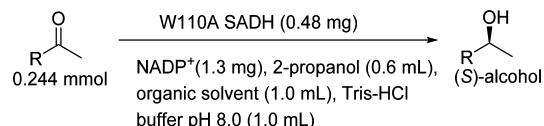


entry	solvent	conv (%)	ee (%)
1	hexane	80	69
2	toluene	24	55
3	diisopropyl ether	37	73
4	<i>tert</i> -butyl alcohol	38	63
5 ^a	Tris-HCl buffer	95	37

^aReaction was conducted using free enzyme in aqueous medium.

enantioselectivity of asymmetric reduction of phenyl-ring-containing ketones using W110A SADH.⁶⁷ We noticed an improvement in ee in the enantioselective reduction of phenylacetone to (*S*)-1-phenyl-2-propanol using W110A SADH when reactions were conducted in media containing water-miscible organic solvents, with the best results obtained when acetonitrile was used, as shown in Table 26. The use of 1-

Table 26. Asymmetric Reduction of Phenyl-Ring-Containing Ketones by W110A SADH in Nonaqueous Media



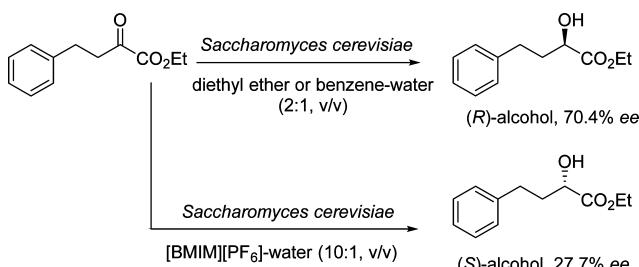
entry	R	solvent	conv (%)	ee (%)
1	PhCH ₂	[BMIM][BF ₄]	88	38
2	PhCH ₂	DMF	97	56
3	PhCH ₂	acetonitrile	90	62
4	PhCH ₂	[BMIM][NTf ₂]	>99	60
5 ^a	PhCH ₂		95	37
6	4-MeOC ₆ H ₄ (CH ₂) ₂	[BMIM][BF ₄]	40	87
7	4-MeOC ₆ H ₄ (CH ₂) ₂	DMF	35	86
8	4-MeOC ₆ H ₄ (CH ₂) ₂	acetonitrile	28	94
9	4-MeOC ₆ H ₄ (CH ₂) ₂	[BMIM][NTf ₂]	52	88
10 ^a	4-MeOC ₆ H ₄ (CH ₂) ₂		87	91

^aSee ref 36a.

butyl-3-methylimidazolium bis((trifluoromethyl)sulfonyl)imide ([BMIM][NTf₂]), a water-immiscible ionic liquid, leads to a comparable enhancement in enantioselectivity. No such improvement is noticed when a water-miscible ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF₄]), was used. Similar results were obtained in the asymmetric reduction of 4-(4'-methoxyphenyl)-2-butanone to its corresponding *S* alcohol. It was noticed that yield enhancement was observed in both the reduction and oxidation pathways of W110A SADH-catalyzed transformations when [BMIM]-[NTf₂] was used as the nonaqueous medium, thus demonstrating that the partition coefficients of the cosolvents between aqueous medium and [BMIM][NTf₂] are not the only factors influencing the percent conversion in such biphasic systems.

Shi et al. reported the influence of [BMIM][PF₆], a water-immiscible ionic liquid, and water-immiscible organic solvents on the enantioselectivity of the reduction of ethyl 2-oxo-4-phenylbutyrate by *S. cerevisiae* (Scheme 1).⁶⁸ They noticed that

Scheme 1. Asymmetric Reduction of 2-Oxo-4-phenylbutyrate by *S. cerevisiae* ADH in Organic Solvents and [BMIM][PF₆]



(R)-ethyl 2-hydroxy-4-phenylbutyrate was obtained in 70.4% ee when benzene or diethyl ether was used as the solvent; however, a shift to the *S*-configured product in 27.7% ee was noticed when [BMIM][PF₆] was used. Addition of water ([BMIM][PF₆]/water, 10:1, v/v) resulted in a shift to the *R*-configured alcohol (7% ee) that was increased to 82.5% ee by addition of ethanol (1%, v/v).

Zhu and co-workers reported that both conversion and enantioselectivity of SsCR-catalyzed reduction of 4-chlorobenzophenone and 4-methylbenzophenone to their corresponding (R)-alcohols were dependent on the reaction medium, as shown in Table 27.⁴⁶ For both substrates, the best conversion

Table 27. Enantioselective Reduction of 4-Chlorobenzophenone and 4-Methylbenzophenone Using Wild-Type SsCR in Reaction Media with Different Organic Cosolvents^a

cosolvent	4-chlorobenzophenone		4-methylbenzophenone	
	conv (%)	ee (%)	conv (%)	ee (%)
no solvent	47	50	45	82
DMSO	97	70	98	80
2-propanol	93	74	>99	80
methanol	95	78	>99	84
butyl acetate	14	62	11	
MTBE	46	58	52	78
THF	62	88	67	92

^aReactions were performed in phosphate buffer (100 mM, pH 7.0) containing 10% of organic cosolvent.

and enantioselectivity results were obtained when 2-propanol or methanol was used as the cosolvent. The addition of THF as the cosolvent resulted in significant improvement in enantioselectivity of the asymmetric reduction of both substrates; however, increasing the amount of THF to more than 10% (v/v) resulted in low or no conversions. Such high enantioselectivities in production of (R)-4-chlorobenzhydrol and (R)-4-methylbenzhydrol, important precursors for the synthesis of optically active forms of cetirizine hydrochloride and neobenodine, respectively, using SsCR in media an organic cosolvent are remarkable because getting beyond 47% ee using chiral metal catalysts is not possible.

The relationship of the reaction medium to activity and selectivity of ADHs with a physicochemical property of organic solvents has been the subject of interest, but no useful correlations have been found.^{69–71} There is certainly more to be explored in medium engineering to simplify the selection of solvent in a certain enzymatic reaction, which could serve as a

simpler alternative to the time-consuming protein engineering approach.

SUMMARY AND OUTLOOK

Over the course of this review, factors influencing substrate specificity and stereospecificity for ADHs have been discussed. Protein engineering, medium engineering, reaction temperature, reaction pressure, and pH show significant effects on ADH-catalyzed reactions. This review shows the importance of small alterations that could be made through protein engineering of the active site on expanding substrate specificity and stereospecificity. On the other hand, a simple and less laborious approach such as medium engineering can be used to improve the stereospecificity of ADHs. Reaction temperature and pressure have been shown to be important in altering the stereospecificity of ADH-catalyzed reactions. Combining mutagenesis and medium engineering can even further improve reactions, as shown by several examples above. Expanding the substrate specificity and stereospecificity of ADHs will improve the effectiveness of redox reactions catalyzed by these enzymes and, thus, make them good alternatives to other environmentally harsh methods. The pharmaceutical industry will probably find more uses in the future for ADHs because of their ability to produce enantiopure alcohols with either desired configuration, whereas typical chemical reaction conditions produce only one enantiomer under more severe conditions.

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Notes

The authors declare no competing financial interest.

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