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Mutation of Cysteine-295 to Alanine in Secondary Alcohol Dehydrogenase from *Thermoanaerobacter ethanolicus* Affects the Enantioselectivity and Substrate Specificity of Ketone Reductions

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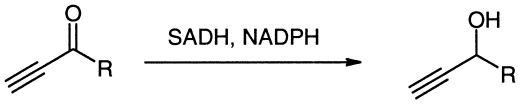
Abstract—The mutation of Cys-295 to alanine in *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase (SADH) was performed to give C295A SADH, on the basis of molecular modeling studies utilizing the X-ray crystal structure coordinates of the highly homologous *T. brockii* secondary alcohol dehydrogenase (1YKF.PDB). This mutant SADH has activity for 2-propanol comparable to wild-type SADH. However, the C295A mutation was found to cause a significant shift of enantioselectivity toward the (*S*)-configuration in the reduction of some ethynylketones to the corresponding chiral propargyl alcohols. This result confirms our prediction that Cys-295 is part of a small alkyl group binding pocket whose size determines the binding orientation of ketone substrates, and, hence, the stereochemical configuration of the product alcohol. Furthermore, C295A SADH has much higher activity towards *t*-butyl and some α-branched ketones than does wild-type SADH. The C295A mutation does not affect the thioester reductase activity of SADH. The broader substrate specificity and altered stereoselectivity for C295A SADH make it a potentially useful tool for asymmetric reductions.

Alcohol dehydrogenases (ADHs) have been used extensively for the asymmetric synthesis of chiral building blocks because of their high chemoselectivity, enantioselectivity, tolerance of a wide spectrum of functional groups, and their relative ease of use.[1] A number of ADH's have been isolated from thermophilic organisms, the best studied being that from *Thermoanaero bacter brockii* (TBADH)[2] Due to their high stability to heat, organic co-solvents, and immobilisation, these enzymes have received considerable attention in chiral synthesis. Although thermostable ADHs have been shown to reduce many different ketones bearing a variety of other functionalities enantioselectively to the corresponding secondary alcohols, there is still a need to further explore their synthetic scope and to deepen our knowledge of the physical principles of their stereochemistry.



In an earlier study,[3] we investigated the asymmetric reduction of ethynyl ketones and ketoesters by secondary alcohol dehydrogenase (SADH) from *T. ethanolicus* [eq (1)], and we found that trifunctional chiral building blocks are obtained in good yield and excellent enantiomeric excess. We also showed that although ethynylketones are substrates for SADH and the stereospecificity of their reduction clearly follows the established 'pocket'-model,[4,5] these α,β-unsaturated ketones also irreversibly inactivate the enzyme, which requires the use of relatively large enzyme concentrations to obtain good conversion. We had postulated that a nucleophilic residue in the active site might cause the inactivation by adding to the electron-deficient triple bond in the ethynylketone substrates. This result stimulated us to perform modeling studies of SADH using the available coordinates of the highly homologous enzyme, TBADH. The result of these modeling studies predicted

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that Cys-295 is located in the postulated 'small alkyl group' binding pocket. In the present study, we investigated the effect of the mutation Cys295 → Ala (C295A) on the enantioselectivity of ethynylketone reduction and on substrate specificity of SADH.

Results and Discussion

We examined the X-ray crystal structure[6,7] of TBADH (1YKF.PDB), which is very highly homologous (99% identity) to SADH, in order to find the location and orientation in which the substrates bind to the enzyme. Only one conformation of a good substrate,[3] isopropyl 4-oxo-5-hexynoate, was found that was free from severe steric overlaps upon docking in the active site. In this structure, presented in Figure 1, the ethynyl group protrudes into a region enclosed by the residues, His-59, Ala-85, Ile-86, Asp-150, Thr-153, and Cys-295. Thus, these residues make up the previously proposed 'small alkyl binding pocket'.[4,5] The alkyl chain bearing the ester group occupies a space surrounded by Cys-37, Ser-39, Leu-107, Trp-110, Tyr-267, Gly-293, Leu-294, and the nicotinamide ring of the cofactor. Cys-37, His-59 and Asp-150, which are conserved in all sequences of SADH and related enzymes (bold italic characters, Fig. 2), are ligands to the catalytic zinc ion (Fig. 1). The other residues located in the small pocket, Ala-85, Ile-86, Thr-153, and Cys-295 are only partially conserved in related sequences (bold characters, Fig. 2). After our studies had been completed, the X-ray structure of the apoenzyme of TBADH with bound (*S*)-2-butanol (1BXZ.PDB) was published,[8] and a model for DMSO bound to TBADH was proposed.[9] The substrate binding site presented in these papers is very similar to our model shown in Figure 1; however, the potential role of Cys-295 in substrate binding was not recognized.

The nicotinamide ring is also very close in space to the α-hydrogens of the substrate in our model (Fig. 1). This observation explains why any branching in this position precludes binding in this orientation, as can be seen not only in the lack of reactivity of 4-methyl-1-heptyn-3-one (**8** in Table 1), but also in the high enantiomeric excess obtained in the reduction of 4-methyl-1-pentyn-3-one (**2** in Table 1) by wild-type SADH. The model places the ester carbonyl oxygen of isopropyl 4-oxo-5-hexynoate within hydrogen bonding distance of Ser-39, and although no significant difference in enantiomeric excess or yield between ethynylketoesters and simple ethynylketones of comparable size were found, we have found that the ketoesters react much faster than the simple ketones.[3] Moreover, this result suggests that hydrogen bonding may take place between Ser-39 and

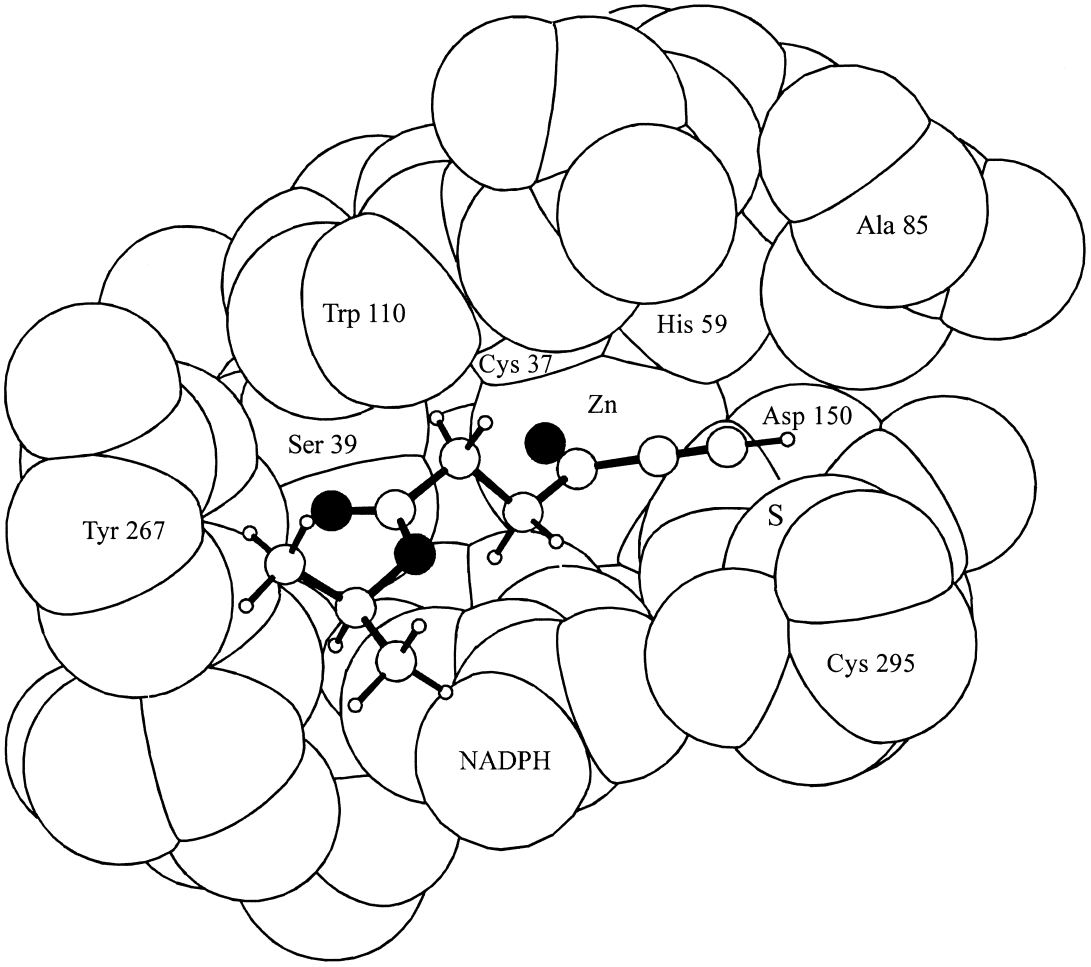

Figure 1. Active site model of wild-type SADH with bound substrate, isopropyl 4-oxo-5-hexynoate. Coordinates were taken from 1YKF.PDB.

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S71131       1 ~~~~MKGFAMLSIGKVGWIEKEKPAPGPFDAIVRPLAVAPCTSDIHTVFEGAIGERHNMI
P14941       1 ~~~~MKGFAMLSIGKVGWIEKEKPAPGPFDAIVRPLAVAPCTSDIHTVFEGAIGERHNMI
P25984       1 ~~~~MKGFAMLGINKLGWIEKERPVAGSYDAIVRPLAVSPCTSDIHTVFEGALGDRKNMI
P35630       1 ~~~~MKGLAMLGIGRIGWIEKKIPECGPLDALVRPLALAPCTSDTHTVWAGAIGDRHDMI
P75214       1 ~~~~MKAYAMLKIGATGWIEKPRPVCGPNDAIIRPLAVAPCTSDVHTVWEGGIGERHNMV
P14940       1 MTAMMKAAVFVEPGRIELADKPIPDIGPNDALVRITTTTICGTDVH.ILKGEYPVAKGLT
consensus    1 ....**.............*..*..*..**..*......*..*.*....*.........

S71131      57 LGHEAVGEVVEVGSEVKDFKPGDRVVVPAITPDWWTSEVQRGYHQHSGGML.........
P14941      57 LGHEAVGEVVEVGSEVKDFKPGDRVVVPAITPDWRTSEVQRGYHQHSGGML.........
P25984      57 LGHEAVGEVVEVGSEVKDFKPGDRVIVPCTTPDWRSLEVQAGFQQHSNGML.........
P35630      57 LGHEAVGQIVKVGSLVKRLKVGDKVIVPAITPDWGEEESQRGYPMHSGGML.........
P75214      57 LGHEGCGVVDEVGSEVKSFKVGDRVLVAAITPEWNSVNAQAGYPMHSGGML.........
P14940      60 VGHEPVGIIEKLGSAVTGYREGQRVIAGAICPNFNSYAAQDGVASQDGSYLMASGQCGCH
consensus   61 .***..*.....**.*.....*..*......*.......*.*........*.........

S71131     108 .....AGWKFSNVKDGVFGEFFHVNDADMNLAHLPKEIPLEAAVMIPDMMTTGFHGAELA
P14941     108 .....AGWKFSNVKDGVFGEFFHVNDADMNLAHLPKEIPLEAAVMIPDMMTTGFHGAELA
P25984     108 .....AGWKFSNFKDGVFGEYFHVNDADMNLAILPKDMPLENAVMITDMMTSGFHGAELA
P35630     108 .....GGWKFSNFKDGVFSEVFHVNEADANLALLPRDIKPEDAVMLSDMVTTGFHGAELA
P75214     108 .....GGWKFSNVKDGMFAEYFHVNDAEGNLALMPEGMDLADACMLSDMIPTGFHANELA
P14940     120 GYKATAGWRFGNMIDGTQAEYVLVPDAQANLTPIPDGLTDEQVLMCPDIMSTGFKGAENA
consensus  121 ......**.*.*..**...*...*..*..**...*.........*..*....**...*.*

S71131     163 DIELGATVAVLGIGPVGLMAVAGAKLRGAGRIIAVGSRPVCVDAAKYYGATDIVNYKDGP
P14941     163 DIELGATVAVLGIGPVGLMAVAGAKLRGAGRIIAVGSRPVCVDAAKYYGATDIVNYKDGP
P25984     163 DIQMGSSVVVIGIGAVGLMGIAGAKLRGAGRIIGVGSRPICVEAAKFYGATDILNYKNGH
P35630     163 NIKLGDTVCVIGIGPVGLMSVAGANHLGAGRIFAVGSRKHCCDIALEYGATDIINYKNGD
P75214     163 DIQYGVALSFFCAGPVGLMAIAGAALKGAGRIIVVDSRPDIVEIAKAYGATDYIDFKKVS
P14940     180 NIRIGHTVAVFAQGPIGLCATAGARLCGATTIIAIDGNDHRLEIARKMGADVVLNFRNCD
consensus  181 .*..*........*..**...***...**..*...........*...**.........

S71131     223 IESQIMNLTEGKGVDAAIIAGGNADIMATAVKIVKPGGTIANVNYFGEGEVLPVPRLEWG
P14941     223 IESQIMNLTEGKGVDAAIIAGGNADIMATAVKIVKPGGTIANVNYFGEGEVLPVPRLEWG
P25984     223 IVDQVMKLTNGEGVDRVIMAGGGSETLSQAVSMVKPGGIISNINYHGSGDALLIPRVEWG
P35630     223 IVEQILKATDGKGVDKVVIAGGDVHTFAQAVKMIKPGSDIGNVNYLGEGDNIDIPRSEWG
P75214     223 VVDEILKWTNNEGVEKVLISGGGSTILETAIKVLRPGGKIGNVNYFGAGEFLTIPRVEWG
P14940     240 VVDEVMKLTGGRGVDASIEALGTQATFEQSLRVLKPGGTLSSLGVYSSD..LTIPLSAFA
consensus  241 ........*...**.......*............**................*.....

S71131     283 CGMAHKTIKGGLCPGGRLRMERLIDLVFYKPVDPSKLVTHVFQGFDNIEKAFMLMKDKPK
P14941     283 CGMAHKTIKGGLCPGGRLRMERLIDLVFYKRVDPSKLVTHVFRGFDNIEKAFMLMKDKPK
P25984     283 CGMAHKTIKGGLCPGGRLRAEMLRDMVVYNRVDLSKLVTHVYHGFDHIEEALLLMKDKPK
P35630     283 VGMGHKHIHGGLTPGGRVRMEKLASLISTGKLDTSKLITHRFEGLEKVEDALMLMKNKPA
P75214     283 VGMAHKAIHGGLMLGGRLRLEKLARLIMTKKLDPSKMITHRFKGFEHIEEALFLMKDKPK
P14940     298 AGLGDHKINTALCPGGKERMRRLINVIESGRVDLGALVTHQYR.LDDIVAAYDLFANQRD
consensus  301 .*.....*...*..**..*...*.........*.....**.........*..*......

S71131     343 DLIKPVVILA~~~~~~~~
P14941     343 DLIKPVVILA~~~~~~~~
P25984     343 DLIKAVVIL~~~~~~~~~
P35630     343 DLIKPVVRIHYDDEDTLH
P75214     343 DLIKSVVIF~~~~~~~~~
P14940     357 GVLKIAIKPH~~~~~~~~
consensus  361 ...*............
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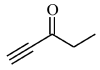
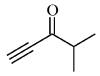
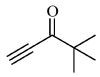
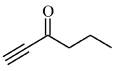
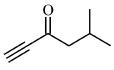
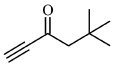
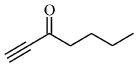
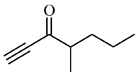
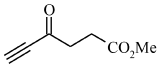
Figure 2. Alignment of SADH from *T. ethanolicus* and related sequences. S71131: *T. ethanolicus*; P14941, *T. brockii*; P25984, *Clostridium beijerinckii*; P35630, *Entamoeba histolytica*; P75214, *Mycoplasma pneumoniae*; P14940, *Alcaligenes euthrophus*. The zinc ligands are shown in italics, and the residues which comprise the small alkyl binding pocket are shown in bold.

Table 1. C295A SADH reductions of ethynylketones

Entry	Substrate	Yield[a,b] (%)	Abs. conf.[a]	ee.[a] (%)
1		39 (32)	*S* (*S*)	76 (80)
2		88 (50)	*S* (*S*)	>98 (>98)
3		39 (0)	*S* (*S*)	85 (85)
4		51 (28)	*S* (*S*)	76 (51)
5		42 (20)	*S* (*R*)	56 (50)
6		0 (0)	—(*R*)	—(66)
7		60 (32)	*S* (*R*)	67 (42)
8		43 (0)	*S* (–)	>98 (–)
9		23 (35)	*R* (*R*)	60 (82)

[a]Absolute configuration. Results of reductions with wild-type SADH are given in parentheses.
[b]Yields reported are isolated yields.

the amide nitrogen in the proposed physiological substrate of SADH, acetyl CoA.[10] Our previous finding that the mutation Ser-39→Thr shifts the enantiospecificity for 2-butanol and 2-pentanol toward the (*R*)-enantiomers supports our conclusion that Ser-39 is part of the large pocket in SADH.[11] We hypothesized that Cys-295 may be responsible for the irreversible inactivation of the enzyme observed during reaction with some ethynyl ketones.[3] If so, replacement of Cys-295 with a non-nucleophilic residue by site-directed mutagenesis should therefore abolish the observed inactivation. Cys-295 is conserved in some, but not all, related ADH sequences. In the ADH from *Entamoeba histolytica*, it is replaced by threonine, and in that from *Mycoplasma pneumoniae*, it is replaced with methionine (Fig. 2). We chose the Cys-295→Ala mutation as the least disruptive mutation to test this hypothesis. This mutation should furthermore result in an enlargement of the small alkyl binding pocket, due to the absence of the large sulfur atom, which has a steric bulk comparable to a CH_2, a change which should be reflected in a change in substrate specificity and enantioselectivity. Since a larger 'small pocket' would accommodate larger alkyl substituents, an overall shift of enantioselectivity towards (*R*)-alcohols for simple alkyl ketone reduction and (*S*)-alcohols for ethynyl ketone reduction should entail if the model shown in Figure 1 is correct.

The specific activity of C295A SADH (43 U/mg) is only slightly lower than that of the wild-type enzyme (54 U/mg)[12] indicating that the mutation does not disrupt the active site structure. We reduced a series of ethynyl ketones with SADH C295A in order to compare the outcome with our previous studies with wild-type SADH[3]. However, the ethynylketones were found to inactivate the mutant enzyme in the same manner and extent as was found with wild-type SADH (data not shown). Thus, Cys-295 does not appear to be involved in the mechanism of inactivation. It is possible that the inactivation is due to reaction of the zinc ligand, Cys-37, with the ethynylketones. Alternatively, Peretz et al. reported that TBADH is inactivated by sulhydryl reagents reacting at Cys-203.[13]

We observed a significant effect of the C295A mutation on the enantioselectivity of the reductions, as can be seen in Table 1. These reactions were performed with the goal of optimizing chemical yield rather than enantioselectivity; thus, the reactions cannot be compared quantitatively. Nevertheless, the comparison of the outcome of the reactions of wild-type and C295A mutant SADH under otherwise identical conditions is of practical interest. It is apparent that the stereochemical outcome for small substrates is largely unaffected (entries **1**–**3**), likely because the the reactions are governed primarily by the degree of steric interaction with the large pocket (i.e., the nicotinamide ring) rather than by the size of the small pocket, which can easily accommodate any of the substituents in this group. With wild-type SADH, 4-methyl-1-pentyne-3-one (**2** in Table 1) exhibits very high enantioselectivity for reduction. We propose that this is due both to the ability of the small alkyl pocket to comfortably accommodate the isopropyl group as well as to the unfavorable interactions of the α-branching for binding the isopropyl group in the large pocket. There is no significant difference in the result of reduction of **2** with wild-type or C295A mutant SADH (Table 1). However, the yield of product (but not the ee) from reduction of a *t*-butyl ketone (**3**) is much higher for C295A SADH (Table 1). The steric interaction with the cofactor should be smallest with the ethynyl group, due to its linear geometry, hence the preference for the binding orientation that leads to the (*S*)-enantiomers. The substrates of intermediate size (**5** and **7** in Table 1), however, experience a reversal of preferred configuration of the product from (*R*) for wild-type to (*S*) for C295A SADH, indicating that the small pocket in the mutant SADH is able to accommodate significantly larger alkyl groups than wild-type SADH. Furthermore, substrate **4** shows a marked increase in stereoselectivity of reduction with C295A SADH. The most striking evidence for the importance of the steric interactions of α-substituents with the dihydronicotinamide ring comes from compound **8**, 4-methyl-1-heptyn-3-one, which does not react at a significant rate with wild-type SADH. In contrast, **8** is rapidly reduced by C295A SADH to give a 2:1 mixture of (3*S*,4*S*) and (3*S*,4*R*)-diastereomers in good chemical yield, with an enantiomeric excess for each diastereomer greater than 98%. Thus, the 'small alkyl' pocket is now large enough to accomodate even the *sec*-pentyl group,

Table 2. Kinetic parameters of thioester reduction by wild-type and C295A SADH

Compound	SADH	K_m ($\times 10^{-3}$ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})
Ethylthioacetate	Wild-type	6.0	37	6.2
Ethylthioacetate	C295A	14	38	2.7
Acetyl–CoA	Wild-type	0.6	73	122
Acetyl–CoA	C295A	1.2	50	42

whereas binding of this group in the large pocket is still precluded because of the severe steric interactions between NADPH and the α-methyl group in this substrate. As the size of the alkyl substituent is increased further, the influence of the Cys to Ala mutation decreases substantially, so that an ethynyl ketoester substrate, **9**, reacts similarly with wild-type and mutant SADH.

Burdette et al.[10] found that SADH from *T. ethanolicus* acts as a thioester reductase, reducing the acetyl group of acetyl CoA to give a thiohemiacetal, which releases CoA and acetaldehyde, which is further reduced to ethanol. Hence, it was suggested that acetyl CoA is the physiological substrate for SADH. Within this context, we examined whether Cys-295 is required for the thioesterase mechanism of SADH. If so, the thioester reductase activity should be greatly reduced or abolished in C295A SADH. However, the kinetic parameters for both ethyl thioacetate and acetyl CoA show only a small difference between wild-type and C295A SADH (Table 2). These differences are not significant enough to indicate any involvement of Cys-295 in the chemical mechanism of thioester reduction. It is interesting to note that acetyl CoA is a significantly better substrate than ethyl thioacetate, with comparable k_{cat} values but K_m values about 10-fold lower (Table 2). This result is in accord with our finding that longer chain ketoesters such as isopropyl 4-oxo-5-hexynoate are better substrates for reduction than are alkyl ketones.[3]

Conclusions

The results presented herein provide a good indication of the location and orientation of substrates in the active site of SADH. The residues that comprise the large and small alkyl binding pockets have been clearly identified by modeling studies and confirmed by our experimental results. These results show that modification of the side chains making up the small alkyl binding pocket by site-directed mutagenesis results in a significant change in both substrate specificity and in enantioselectivity of ketone reduction without significant loss of activity. Starting with this active-site model, together with our previous results from the mutation of Ser39→Thr, further mutations in the small pocket could be envisioned to result in even higher specificity for (*R*)-alcohols. C295A SADH has broader substrate specificity for α-branched ketones than wild-type SADH and TBADH, making it a potentially useful tool in asymmetric reductions.

Experimental

General

Enzyme assays and kinetic experiments were performed on a Varian Cary 1E UV–visible spectrophotometer equipped with a Peltier-type thermoelectric temperature-controlled 6×6 cell cuvette changer. Capillary GC was performed on a Varian 3300 gas chromatograph with FI detection (Supelco β-Dex 120 chiral column, 30 m×0.250 mm id, 0.25 μm film thickness) programmed between 40 and 175 °C. ^1H and ^{13}C NMR spectra were taken on a Bruker AC-250 or AC-300 spectrometer, respectively, using the residual $CHCl_3$ signal (δ 7.26 ppm) as internal reference. Optical rotations were measured on a Rudolph Autopol IV polarimeter.

Enzyme assay

SADH was assayed at 50 °C by following the increase in absorbance at 340 nm due to the formation of NADPH ($\Delta\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$).[5] The assay mixture contained 200 mM 2-propanol and 1.25 mM NADP in 50 mM Tris–HCl buffer, pH 8.0. One unit (U) is the amount of enzyme required to reduce 1 μmol of NADP per minute.

Modeling studies of SADH

Low-energy conformations of isopropyl 4-oxo-5-hexynoate, an excellent substrate that gives very high ee of (*R*)-alcohol upon reduction,[3] were identified using the Tripos force-field (Sybyl 6.3, Tripos Inc., 1996). All waters were removed from the protein structure (1YKF.PDB), and these ketones were then docked into the active site using the Sybyl 6.3 program in such a way that the carbonyl oxygen was in close proximity to the catalytic zinc ion and to the *pro*-R hydrogen in the 4-position of the nicotinamide ring of the NADPH cofactor. The substrate was oriented such that hydride transfer would occur on its *si*-face, since we had observed very high stereoselectivity for (*R*)-alcohol in the reaction. In order to accommodate the substrate, it was necessary to rotate the nicotinamide ring about the C–N bond between the pyridine nitrogen and the ribose-C1. A similar rotation was performed by Kleifeld et al.[9] in modeling the complex of TBADH and DMSO. Comparison of the HLADH structures with and without DMSO bound had shown that a rotation of the nicotinamide ring occurs on ligand binding.[14,15] After thus obtaining reasonable starting structures, these were then energy-minimized using the TRIPOS force-field and examined for validity based on the absence of steric overlap.

Sequence alignments of SADH

The amino acid sequence of SADH was used as a query for a PSI-BLAST search in the *nr* database at NCBI. Those sequences with highest similarities were then aligned using the PILEUP program of GCG.[16] For display, the output from the PILEUP program was run with BOXSHADE to generate Figure 2.

Preparation of C295A mutant SADH

Point mutations were introduced as described previously.[17] PCR-based *T. ethanolicus* 39E *adh*B gene mutagenesis was performed using plasmid pADHB25-kan as template. An oligonucleotide primer (KA4N) was synthesized complementary to the noncoding strand that included a *Kpn*I restriction site, the native *adh*B ribosome binding site, and the *adh*B translation initiation codon (5′-CGGGGTACCCCGTATTTTA-GGAGGTGTTTAATGATGAAAGG-3′). A second roligonucleotide primer (KA4C) that included the complement to the *adh*B termination codon and the *Apa*I estriction site was synthesized complementary to the coding strand (5′-CAGTCCGGGCCCTTATGCTAATAT-TACAACAGGTTTG-3′). Complementary 33 oligonucleotide primers that contained the mutated bases (5′-CTATAAAAGGCGGGCTAGCCCCCGGT-GGACGTC-3′) and (5′-GACGTCCACCGGGGGC-TAGCCCGCCTTTTATAG-3′) were used together with the two KA4 end primers to amplify the 5′ and 3′ ends of the *adh*B gene. PCR synthesis of mutated partial and complete *adh*B gene were performed using TagPlus Precision™ PCR system (Stratagene, LA Jolla, CA). Obtained constructs were subcloned in the *Kpn*I and *Apa*I restricted pBluescriptII KS (±) and expressed in *Eschericlia coli* DH5α. Mutations were verified by whole gene sequencing using the Thermo Sequenase™ radiolabeled terminator cycle sequencing kit (USB Corporation, Cleveland, OH, USA). DNA work was performed using published protocols.[18,19]

Purification of C295A SADH

For asymmetric reductions. *E. coli* cells expressing C295A SADH were grown as described.[17] The wet cells (12 g) were suspended in 50 mM Tris–HCl buffer, pH 8.0, (50 mL) containing 0.1 mM dithiothreitol, sonicated, and centrifuged (30 min at 10000×*g*, all of the following centrifugations were done under these conditions). The supernatant was heated in a capped bottle at 70 °C for 1 h, and the thick suspension was then centrifuged. This heat treatment was repeated once more, whereupon the supernatant had an activity of 92 U/ml and a specific activity of 27 U/mg protein.

For determination of kinetic parameters. The enzyme solution obtained above was further purified by affinity chromatography on Red-Agarose as previously described[11] and gave C295A SADH with a specific activity of 43 U/mg protein.

Preparation of ethynylketones.[20] A solution of acyl chloride (10.0 mmol) and bis-TMS-acetylene (1.87 g, 11.0 mmol) in dry CH_2Cl_2 (25 mL) was cooled to 0 °C and $AlCl_3$ (1.60 g, 12.0 mmol) was added in portions during 30 min. The mixture was stirred for 2 h at 0 °C and for 2 h at room temperature and then poured into a 1:1 mixture of 1 N HCl and ice (50 mL). The layers were separated and the aqueous phase extracted with CH_2Cl_2 (3×30 mL). The combined organic extracts were dried with Na_2SO_4 and the solvent was removed in vacuo. The residue was taken up in Et_2O (40 mL) and the resulting solution was stirred vigorously overnight with an aqueous solution of NaF (0.462 g, 11.0 mmol) and NBu_4Cl (0.31 g, 1.1 mmol). The layers were separated and the aqueous layer extracted with Et_2O (3×25 mL). The combined organic extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue chromatographed on silica gel.

Enzymatic reduction of ethynylketones with SADH C295A

SADH C295A (600 U) and NADP (1 mg, 1.2 μmol) were dissolved in 50 mM Tris–HCl buffer, pH 8.0, (8.5 mL). After preincubation for 10 min at 50 °C, the appropriate ketone (1.0 mmol) was added in one portion and the mixture was kept at 50 °C. As soon as the ketone was consumed (by GC analysis), the reaction mixture was saturated with NaCl and extracted with Et_2O (3×4 mL). Due to their tendency to form emulsions, the extractions had to be centrifuged (10 min at 4000 rpm) prior to separation. The combined extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue chromatographed. The reactions were stopped immediately upon consumption of the ketone, so they were not allowed to reach equilbrium, and the products were at least partially kinetically controlled.

Determination of optical purity of the alcohols

The alcohol (0.02 mmol) was dissolved in $CDCl_3$ (0.3 mL) in an NMR tube, and a 50 mM solution of $Eu(hfc)_3$ in $CDCl_3$ (0.5 mL) was added in portions of 0.1 mL. A ^1H NMR spectrum was taken after each addition, and the two signals corresponding to the diastereotopic carbinol protons were integrated. If only one signal was visible, the enantiomeric excess was assumed to be greater than 98%. Enantiomeric excess was calculated as either %(*R*) −%(*S*) or %(*S*) −%(*R*), depending on which enantiomer was present in greater amount.

(*S*)-1-Pentyn-3-ol. Yield 32.7 mg (39%), 76% ee, R_f 0.20 (10% ether/pet. ether), ^1H NMR (300 MHz, $CDCl_3$) δ 4.32 (dt, J = 6.5 Hz, 2.0 Hz, 1H), 2.46 (d, J = 1.8 Hz, 1H), 1.74 (m, 3H), 1.02 (t, J = 7.4 Hz, 3H).

(*S*)-4-Methyl-1-pentyn-3-ol. Yield 86.3 mg (88%), >98% ee, R_f 0.22 (10% ether/pet. ether), ^1H NMR (300 MHz, $CDCl_3$) δ 4.17 (dd, J = 5.7 Hz, 2.0 Hz, 1H), 2.45 (d, J = 2.3 Hz, 1H), 1.89 (m, 2H), 1.01 (m, 6H).

(*S*)-4,4-Dimethyl-1-pentyn-3-ol. Yield 13.2 mg (22%, based on 53% conversion), 85% ee, R_f 0.22 (10% ether/pet. ether), $[\alpha]_D^{20}$ −10.0° (*c* 1.5, dioxane) (lit.[21] $[\alpha]_D^{20}$ +35.6° (*c* 3, dioxane), 90% ee for (*R*)-enantiomer), ^1H NMR (250 MHz, $CDCl_3$) δ 4.02 (d, J = 2.1 Hz, 1H), 2.45 (d, J = 2.2 Hz, 1H), 1.8 (bs, 1H), 1.00 (s, 9H).

(*S*)-1-Hexyn-3-ol. Yield 48.1 mg (51%), 76% ee, R_f 0.22 (10% ether/pet. ether), $[\alpha]_D^{20}$ −9.9° (*c* 2.5, $CHCl_3$) (lit.[22] $[\alpha]_D^{20}$ +9.0° (*c* 1.0, $CHCl_3$), 75% ee for (*R*)-enantiomer), ^1H NMR (250 MHz, $CDCl_3$) δ 4.39 (dt, J = 6.5 Hz,

2.2 Hz, 1H), 2.47 (d, $J = 2.1$ Hz, 1H), 1.71 (m, 3H), 1.50 (m, 2H), 0.97 (t, $J = 7.0$ Hz, 3H).

(*S*)-5-Methyl-1-hexyn-3-ol. Yield 47.3 mg (42%), 56% ee, R_f 0.20 (10% ether/pet. ether), $[\alpha]_D^{20}$ −16.1° (*c* 2.3, dioxane) (lit.[21] $[\alpha]_D^{25}$ +28.8° (*c* 3, dioxane), 88% ee for (*R*)-enantiomer), ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.42 (dt, $J = 7.4$ Hz, 2.3 Hz, 1H), 2.46 (d, $J = 2.1$ Hz, 1H), 1.87 (m, 1H), 1.60 (m, 3H), 0.95 (d, $J = 6.7$ Hz, 3H), 0.93 (d, $J = 6.8$ Hz, 3 H).

(*S*)-1-Heptyn-3-ol. Yield 67.8 mg (60%), 67% ee, R_f 0.19 (10% ether/pet. ether), $[\alpha]_D^{20}$ −8.5° (*c* 2.6, $CHCl_3$) (lit.[22] $[\alpha]_D^{20}$ −5.5° (*c* 0.9, $CHCl_3$), 70% ee for (*S*)-enantiomer), ^{1}H NMR (250 MHz, $CDCl_3$) δ 4.37 (dt, $J = 7.3$ Hz, 2.1 Hz, 1H), 2.47 (d, $J = 2.1$ Hz, 1H), 1.87 (bs, 1H), 1.72 (m, 2H), 1.41 (m, 4H), 0.92 (t, $J = 7.0$ Hz, 3H).

(*R*)-Methyl 4-Hydroxy-5-hexynoate. Yield 15.8 mg (23%, based on 48% conversion), 60% ee, R_f 0.20 (20% ethyl acetate/hexanes), $[\alpha]_D^{20}$ +7.0° (*c* 1.8, $CHCl_3$), ^{1}H NMR (250 MHz, $CDCl_3$) δ 4.49 (dt, $J = 6.0$ Hz, 2.0 Hz, 1H), 3.69 (s, 3H), 2.55 (m, 2H), 2.49 (d, $J = 2.1$ Hz, 1H), 2.21–1.98 (m, 3H).

(3*S*,4*S*)- and (3*S*,4*R*)-4-Methyl-1-heptyn-3-ol. (±)-4-Methyl-1-heptyn-3-one (100 mg, 0.81 mmol) was reduced by C295A SADH. The reaction was monitored by GC and worked up after 70% conversion. The product was found to be a 2:1 mixture of diastereomers. Starting material [17 mg (17%)] was recovered, and found by GC to be identical to (*R*)-4-methyl-1-heptyn-3-one (see below). Therefore, the major product of the enzyme reaction was (3*S*,4*S*)-4-methyl-1-heptyn-3-ol. Yield 44 mg (43%), >98% ee, R_f 0.19 (10% ether/pet. ether), $[\alpha]_D^{20}$ −10.2° (*c* 2.75, dioxane) ^{1}H NMR (250 MHz, $CDCl_3$) δ 4.28 (dt, $J = 5.5$ Hz, 2.1 Hz, 1H), 2.46 (d, $J = 2.2$ Hz, 1H), 1.85–1.2 (m, 6H), 1.02 and 1.00 (2:1) (d, $J = 6.7$ Hz, 3H), 0.92 (t, $J = 6.9$ Hz, 3H).

Resolution of 2-methylpentanoic acid.[23] Lipase from *Candida rugosa* (Sigma L1745, 750 units/mg,1.0 g), 1-octanol (130 mg, 1.0 mmol), 2-methylpentanoic acid (116 mg, 1.0 mmol), and heptane (10 mL) were combined and shaken at room temperature for 14 h. The reaction progress was followed by TLC (5% ethyl acetate/hexanes containing 0.25% acetic acid, detection by vanillin) R_f (ester) 0.53, (acid) 0.08, (alcohol) 0.03. The enzyme was removed by filtration, the filtrate evaporated, and the components separated by chromatography. Yield of (*S*)-ester, 73.6 mg (64%), yield of (*R*)-acid, 55.0 mg (93%).

(*R*)-4-Methyl-1-heptyn-3-one. (*R*)-2-Methylpentanoic acid (55.0 mg, 0.47 mmol) and KOH (31 mg) were dissolved in MeOH (1.0 mL), allowed to stand for 90 min, and evaporated to dryness. Benzene (2 mL) was added and the mixture stirred for 60 min. At 0 °C, DMF (1 μL) and oxalyl chloride (250 mg, 2 mmol) were added and stirring was continued at room temperature for 2 h. The excess of oxalyl chloride was removed by evaporation with $CHCl_3$. The residue was taken up in CH_2Cl_2, filtered, and treated with bis-(trimethylsilyl)acetylene (80 mg, 0.47 mmol) and, at 0 °C, $AlCl_3$ (80 mg). The mixture was stirred for 2 h at 0 °C and for 2 h at room temperature and then poured into a 1:1 mixture of 1 M HCl and ice. The layers were separated, and the aqueous phase extracted with CH_2Cl_2. The combined organic extracts were dried with Na_2SO_4 and the solvent was removed in vacuo. The residue was taken up in Et_2O and the resulting solution was stirred vigorously overnight with an aqueous solution of NaF (20 mg, 0.5 mmol) and NBu_4Cl (14 mg, 0.05 mmol) in H_2O (2 mL). The layers were separated and the aqueous layer extracted with Et_2O. The combined organic extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue chromatographed on silica gel. Yield 28.2 mg (48%); ee 78%; ^{1}H NMR (250 MHz, $CDCl_3$) δ 3.22 (s, 1H), 2.59 (m, $J = 6.8$ Hz, 1H), 1.79 (m, 1H), 1.39 (m, 3H), 1.19 (d, $J = 7.2$ Hz, 3H), 0.93 (t, $J = 7.2$ Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.1, 80.9, 79.0, 48.1, 34.5, 20.1, 15.5, 13.9.

Preparation of ethyl thioacetate.[24] A mixture of NaOH (4.0 g, 0.1 mol), H_2O (7.5 mL), and ethanethiol (8.9 mL, 0.12 mol) was poured onto cracked ice (50 g). Acetic anhydride (11.8 mL, 0.125 mol) was added with vigorous stirring, which was continued for 5 min, whereby the thioester separated immediately. The product was washed with H_2O (5×5 mL), dried with $MgSO_4$, and distilled, bp 112–114 °C (lit. bp 114–116 °C). Yield, 5.8 g (56%).

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