The Use of an Altered Specificity Engineered Enzyme for Asymmetric Synthesis: Enantioselective Reduction of 4-Methyl-2-oxopent-3-enoic Acid

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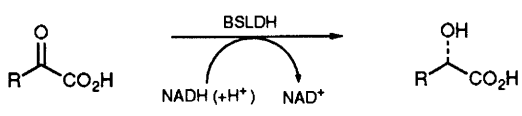
A genetically engineered version of *Bacillus stearothermophilus* lactate dehydrogenase, incorporating structural motifs which serve to alter substrate specificity in favour of α-keto acids with bulky aliphatic side chains, was used to effect enantioselective reduction of 4-methyl-2-oxopent-3-enoic acid.

The provision of small chiral molecules *via* enzymatic transformations is now commonplace. Amongst the reasons for this is the recognition by many synthetic chemists of the practical advantages of using biocatalysts, notably considerations of cost, functional group compatibility and ease of manipulation when compared to auxiliary-based chemical reagents developed for asymmetric synthesis. Although modern genetic methods now make thermostable enzymes available very cheaply, an inherent disadvantage of many enzymes is narrow specificity for unnatural substrates which limits the scope of a particular transformation. One of the ways that this problem is being addressed is by the design and construction of engineered enzymes,[1,2] in which specific amino acid residues within the protein framework are altered in order to accomodate substrates that exhibit little or no reactivity with the corresponding wild-type enzyme. Currently this approach is of limited predictability, and is feasible only with well characterised protein frameworks in which the individual structural features responsible for catalysis and substrate binding have been identified.

Lactate dehydrogenase derived from *Bacillus stearothermophilus* (BSLDH) is ideally suited to genetic manipulation by site-directed mutagenesis.[2] Its sequence is known and considerable information is available on its three-dimensional structure[3] and catalytic mechanism (Fig. 1).[4a] Furthermore, the thermostable wild-type enzyme has been used as a synthetic catalyst for the enantioselective reduction of α-keto acids (eqn. 1) on a preparative scale.[5,6] As part of a long-term research programme aimed at increasing the applicability of



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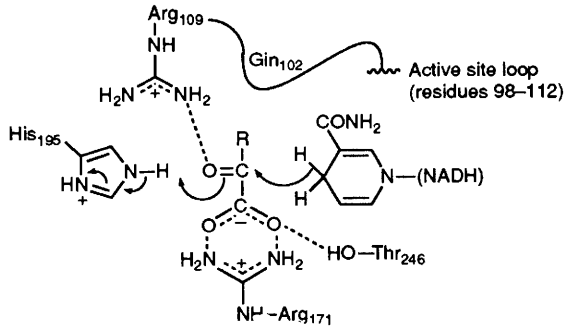


Fig. 1 Schematic representation of the BSLDH reductive mechanism showing some active site residues essential for catalysis[3,4a]

Table 1 Steady-state kinetic parameters for BSLDH-catalysed reduction[a]

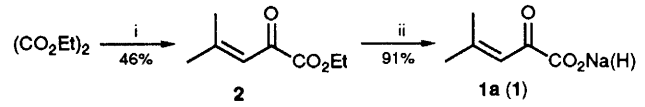
Substrate	Enzyme	k_{cat}/s^{-1}	K_M/ mmol dm^{-3}
$MeCOCO_2H$[b]	Wild-type	250	0.06
	MVS/GG	32	4
$Me_2CHCH_2COCO_2H$[b]	Wild-type	0.33	6.7
	MVS/GG	18.5	14.3
$Me_2C{=}CHCOCO_2H$	Wild-type	(0.03)[c]	—
	MVS/GG	1.2[d]	22[d]

[a] In the presence of 5 mmol dm^{-3} fructose-1,6-bisphosphate. [b] From ref. 7. [c] Turnover when [**1**] = 10 mmol dm^{-3}. [d] Apparent values, from measurements using [**1**] = 1–20 mmol dm^{-3}. (Substrate inhibition was observed at higher concentrations.)

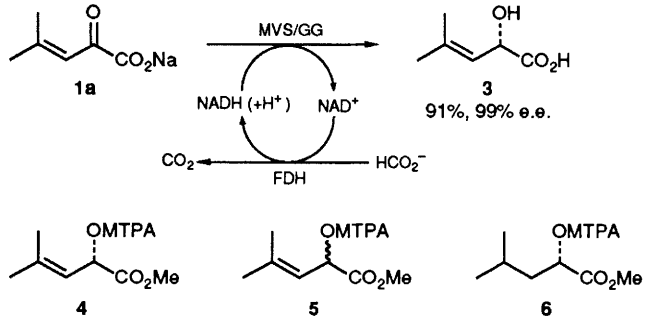
this reaction, mutant versions of BSLDH have been produced, which display enhanced affinity for unnatural substrates whilst retaining the property of high thermostability that is characteristic of the wild-type enzyme.[4b,7] For the improved reduction of α-keto acids with bulky aliphatic side chains, notably the branched substrates 3-methyl-2-oxobutanoate and 4-methyl-2-oxopentanoate, an engineered LDH (MVS/GG) was constructed,[7] containing the following mutations in the two regions of the protein which surround the substrate side chain: (*i*) Gln102Met, Lys103Val and Pro105Ser; to increase hydrophobicity and segmental flexibility of the mobile polypeptide loop (residues 98–112) which folds over the substrate side chain to create a catalytic vacuole. This loop also includes Arg109 which is required for the polarisation of the C-2 carbonyl group of the substrate prior to hydride transfer from NADH. (*ii*) Ala235,236Gly; to generate extra plasticity in the helix onto which the active site loop folds. The combined effect of these mutations is illustrated by kinetic data for the reduction of 4-methyl-2-oxopentanoate (Table 1, entry 2), with a 56-fold increase in catalytic turnover (k_{cat}) compared to wild-type BSLDH.

As an extension to previous studies concerning the reduction of β,γ-unsaturated α-keto acids,[6] we now report our work on the reduction of 4-methyl-2-oxopent-3-enoic acid **1**. The latter was expected to provide a stringent test for the specificity limits of wild-type BSLDH, since its rigid planar structure is likely to exacerbate the deleterious effect of branching at C-4 on redox catalysis. Compound **1** was prepared as the sodium salt **1a** in two steps from diethyl oxalate (Scheme 1). Treatment with 2-methyl-1-propenyl magnesium bromide at −78 °C[8] produced the α-keto ester **2**[9] which was subjected to careful saponification followed by removal of water *in vacuo*.

Steady-state kinetic measurements made under standard conditions[7] indicated that reduction of **1** catalysed by wild-type BSLDH (Table 1, entry 3) proceeded too slowly to measure reliable values of K_M and k_{cat}. As an indication



Scheme 1 *Reagents and conditions*: i, $Me_2C{=}CHMgBr$, THF–Et_2O, −78 °C; ii, 0.1 mol dm^{-3} NaOH–10% EtOH, 20 °C; THF = tetrahydrofuran

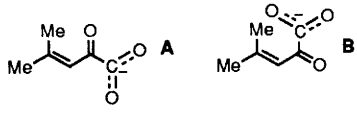


Scheme 2 FDH = formate dehydrogenase

of catalytic efficiency, the turnover of 0.03 s^{-1} at [**1**] = 10 mmol dm^{-3} approximates to a $k_{cat}:K_M$ ratio of <3 dm^3 mol^{-1} s^{-1}. In contrast, reduction of **1** catalysed by the MVS/GG mutant proceeded significantly faster, with catalytic efficiency increased by at least an order of magnitude. The apparent k_{cat} value of 1.2 s^{-1}, although modest when compared to the turnover of pyruvate by wild-type BSLDH, was not expected to be a limitation for preparative-scale work (see below). In an attempt to rationalise these observations, molecular modelling†[a] was used to predict the substrate–protein interactions of **1** with both the wild-type and mutant enzymes. Models were based on the X-ray structure[3] of BSLDH containing NADH and oxamate inhibitor bound in the active site. The anion of substrate **1** was docked in a *cisoid* conformation†[b] by superimposing the carboxy and α-carbonyl groups in the corresponding oxamate positions. Graphic inspection of the wild-type ternary complex after energy minimisation indicated the close proximity of the *cis*-methyl group of **1** with the terminal amide group of the Gln102 side chain. We suggest that this interaction may hinder closure of the active site loop which is essential for redox catalysis. In contrast, the similarly sized methionine residue at position 102 in the MVS/GG mutant relaxes to form a hydrophobic pocket to accommodate the *cis*-methyl group. This effect is probably reinforced by the Ala235,236Gly mutations which provide extra volume inside the catalytic vacuole. The effect of the remaining two mutations (Lys103Val and Pro105Ser) is less obvious, since the side chains in these positions project away from the substrate into solvent. Critically, the geometry of the catalytic centre is maintained in the modelled MVS/GG complex, with the active pro-*R* hydrogen of NADH within 2.7 Å of the α-carbonyl carbon of **1**.

To complete the synthetic aspects of our investigation, enzymatic reduction of compound **1** was carried out preparatively (Scheme 2) with continuous cofactor regeneration *in*

† (*a*) Energy calculations were carried out with the package DISCOVER and graphics inspection and manipulation with INSIGHT, both from Biosym Technologies. Full experimental details will appear in a future account of this work and are also available on written request. (*b*) Energy calculations indicated that the keto carboxylate **1a** exists mainly as the *cisoid* rotamer **A** in solution. Rotamer **B** cannot attain a planar structure due to the proximity of the *cis*-methyl group with a carboxylate oxygen.



situ.[10] MVS/GG (6 U) and yeast formate dehydrogenase (5 U) were added to a solution of **1a** (1.0 mmol) in deoxygenated Tris buffer (5 mmol dm^{-3}; pH 6.0; 80 ml) containing NADH (0.02 mmol), sodium formate (3.1 mmol), fructose-1,6-bisphosphate (0.4 mmol) and dithiothreitol (0.008 mmol). The solution was stirred at room temperature under nitrogen for 5 days, with periodic addition of HCl (0.2 mol dm^{-3}) to maintain pH in the range 6.0–6.2. Acidification to pH 2 and extractive work-up gave (*S*)-2-hydroxy-4-methylpent-3-enoic acid **3** in 91% isolated yield.‡ Analysis of the (*R*)-MTPA [MTPA = α-methoxy-α(trifluoromethyl)phenylacetic acid] (Mosher[11]) derivative **4** and comparison with a racemic standard **5**§ indicated an enantiomeric excess (e.e.) of at least 99% for **3**. Since ^{1}H NMR chemical shift differences between these derivatives were only partly consistent with the expected *S* absolute configuration,[6] additional evidence was obtained by catalytic hydrogenation of **4**, giving an alkanoate derivative **6**§ which was identical to a sample prepared by sequential esterification and *O*-acylation of (*S*)-2-hydroxy-4-methylpentanoic acid.

In conclusion, we have demonstrated the synthetic utility of an engineered enzyme incorporating structural motifs which serve to alter substrate specificity. This study has also enabled the limits of substrate tolerance for the corresponding wild-type enzyme to be defined more clearly. Further work is underway to design and construct new mutants of BSLDH for use as catalysts for asymmetric synthesis.

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‡ Spectroscopically pure material, ν_{max}/cm^{-1} (Nujol) 3360 br and 1721; δ (270 MHz; [$^2H_6$]Me_2CO) 5.23–5.16 (1H, m), 4.81 (1H, d, *J* 8.5 Hz), 1.78 (3H, d, *J* 1.3 Hz) and 1.76 (3H, d, *J* 1.5 Hz); *m/z* 130 (2%; M^+) and 85 (100%; $M^+ - 45$). Recrystallisation (Cl_4C) gave colourless needles, m.p. 85–87 °C, $[\alpha]_D^{20}$ 270+/−10 (*c* 2.3, EtOH). Found: C, 55.24; H, 8.00. $C_6H_{10}O_3$ requires C, 55.39; H, 7.74%.

§ ^{1}H NMR chemical shifts (270 MHz; $CDCl_3$) for CO_2Me protons: 3.75 **4**, 3.79 and 3.75 **5**, 3.75 **6**; CF_3COMe protons: 3.57 **4**, 3.57 and 3.42 **5**, 3.56 **6**. Capillary GC retention times (25 m BPX70 column; 4.5 psi He; 160 °C): 22.2 min **4**, 19.6 and 22.2 min **5**.

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