Strategic manipulation of the substrate specificity of *Saccharomyces cerevisiae* flavocytochrome b_2

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Flavocytochrome b_2 from *Saccharomyces cerevisiae* acts physiologically as an L-lactate dehydrogenase. Although L-lactate is its primary substrate, the enzyme is also able to utilize a variety of other (S)-2-hydroxy acids. Structural studies and sequence comparisons with several related flavoenzymes have identified the key active-site residues required for catalysis. However, the residues Ala-198 and Leu-230, found in the X-ray-crystal structure to be in contact with the substrate methyl group, are not well conserved. We propose that the interaction between these residues and a prospective substrate molecule has a significant effect on the substrate specificity of the enzyme. In an attempt to modify the specificity in favour of larger substrates, three mutant enzymes

have been produced: A198G, L230A and the double mutant A198G/L230A. As a means of quantifying the overall kinetic effect of a mutation, substrate-specificity profiles were produced from steady-state experiments with (S)-2-hydroxy acids of increasing chain length, through which the catalytic efficiency of each mutant enzyme with each substrate could be compared with the corresponding wild-type efficiency. The Ala-198→Gly mutation had little influence on substrate specificity and caused a general decrease in enzyme efficiency. However, the Leu-230→Ala mutation caused the selectivity for 2-hydroxyoctanoate over lactate to increase by a factor of 80.

INTRODUCTION

Saccharomyces cerevisiae flavocytochrome b_2 is a soluble L-lactate dehydrogenase found in the mitochondrial intermembrane space. It is a tetrameric protein, each subunit consisting of an amino acid chain 511 residues in length. Its X-ray-crystal structure, solved to 2.4 Å (0.24 nm) resolution [1], identifies two distinct domains within each subunit. The largest of these contains the enzyme's active site, of which FMN is an integral component. The flavin-binding domain is connected via a short hinge region to a smaller haem-binding domain which functions as an electron-transfer mediator, and as such passes electrons from the catalytically essential FMN to the physiological electron acceptor, cytochrome *c*, direct transfer being prohibited [2,3]. The substrate range of the enzyme is limited to (S)-2-hydroxy acids, many of which are efficiently oxidized to their 2-keto derivatives in the active site (Scheme 1) [4]. The key residues involved in catalysis have been pinpointed in the crystal structure (Figure 1), and a carbanion mechanism proposed [4,5].

The aim of this work is to investigate the origins of substrate specificity amongst 2-hydroxy acids, which lies in favour of L-lactate, and to examine the possibility of its strategic manipulation using site-directed mutagenesis.

Steady-state kinetic analysis of the wild-type enzyme with a series of substrates of increasing chain length has enabled a substrate-specificity profile to be developed, which describes how enzyme efficiency varies with substrate size. Furthermore, close examination of the active-site structure (Figure 2), coupled with sequence comparisons of *S. cerevisiae* flavocytochrome b_2 with related enzymes (Figure 3), has allowed two residues to be identified as being most likely to control this selectivity, these being Ala-198 and Leu-230. In an attempt to modify the selectivity of the enzyme in favour of larger substrates, three mutant enzymes have been constructed: Ala-198→Gly (A198G), Leu-230→Ala (L230A) and the double mutant A198G/L230A.

All three mutants have been characterized by steady-state kinetics, and substrate-specificity profiles developed.

MATERIALS AND METHODS

DNA manipulation, strains and growth

Site-directed mutagenesis was performed by the Kunkel method of non-phenotypical selection [6] using the oligonucleotides 347N (CTGCTAACAGGTTTGTGTAAAC) and 346N (GATATCT-ACTGCTGCTTCATGT) for the A198G and L230A mutations respectively and the single-stranded plasmid, pGR401 [7], as template. A double mutant was also constructed using both oligonucleotides listed above. The oligonucleotides were prepared by Oswel DNA Service, University of Edinburgh, Edinburgh, Scotland, U.K. Mutants were subjected to DNA sequence analysis of the entire flavocytochrome b_2 -coding region to ensure the absence of unwanted secondary mutations. The A198G, L230A and A198G/L230A mutant sequences were transferred into the expression vector pDSb2 [8] by replacing the wild-type flavocytochrome b_2 -coding sequence. Standard methods for growth of *Escherichia coli*, plasmid purification, DNA manipulation and transformation were used as described previously [9].

Enzymes

Wild-type and mutant flavocytochromes b_2 expressed in *E. coli* were isolated from cells, which had been stored at -20°C , using a previously reported purification procedure [8]. Purified enzyme samples were stored under nitrogen at 4°C as precipitates from 70 % -satd. $(\text{NH}_4)_2\text{SO}_4$ solution.

Purified mutant enzymes were demonstrated to be 'intact' rather than 'cleaved' by SDS/PAGE [10], and to be in possession of a full complement of flavin. This was shown by directly measuring the amounts of dissociated flavin and protein-bound

Scheme 1 (S)-2-Hydroxy acid dehydrogenation

R is the only variable in the catalytic dehydrogenation reaction performed by flavocytochrome b_2 . For the physiological substrate L-lactate, R is $-\text{CH}_3$.

Figure 1 Schematic diagram of the flavocytochrome b_2 active site

The diagram is based on the X-ray-crystal structure of the enzyme, which identifies active-site residues. A substrate molecule (L-lactate) is shown in its appropriate binding orientation. FMN, although not shown in this diagram, lies in a parallel plane directly beneath the His-373 residue and substrate molecule.

haem separated on a Sephadex G-25 gel-filtration column (1.5 cm $\times$ 15 cm; Sigma), equilibrated and eluted with Caps (3-cyclohexylamino-1-propanesulphonic acid) buffer (Sigma), pH 11, 1.0 mol/l at 4 °C in darkness, using their known visible absorption maxima [11,12]. The Caps buffer consisted of 0.01 M NaOH titrated against Caps solution to pH 11 and adjusted to 1.0 mol/l using NaCl.

Kinetic analysis

All steady-state kinetic measurements were performed at 25.0 ± 0.1 °C in Tris/HCl buffer, pH 7.5, 1.0 mol/l. The buffer

	198		230		
	↓		↓		
Scb2	PFYVSATALC	KLGNPLEGEK	DVARGCGQGV	TKVPQMISTL	ASCSPEEIEE
Hab2	PFYISATALA	KLGHPEGEV	AIKAGAGRE.	.DVVQMISTL	ASCSFDEIAD
Gox	PIMIAPTAMQ	KMAHP.EGEY	ATARAASAA.	.GTIMTLSSW	ATSSVEEVAS
Bao	PICISPTAFH	SIAWP.DGEK	STARAAQEA.	.NICYVISSY	ASYSLEDIVA
Mdh	PLLIPTGLN	GALWP.KGDL	ALARAATKA.	.GIPFVLSTA	SNMSIEDLAR
Lox	PMFFAPIGVI	ALC.AQDGHG	DAASAQASAR	TGVPYITSTL	AVSSLEDIRK
LctD	PVALAPVGLC	GMYP.RGEV	QAQAADAH.	.GIPFTLSTV	SVCPIREVA.

Figure 3 Sequence comparison of flavocytochromes b_2 and other 2-hydroxy acid dehydrogenases

The sequence of *S. cerevisiae* flavocytochrome b_2 (Scb2) is shown between residues 191 and 240, and is aligned with the corresponding sequences from *Hansenula anomala* flavocytochrome b_2 (Hab2), spinach glycolate oxidase (Gox), rat hydroxy acid oxidase (Hao), *Pseudomonas putida* mandelate dehydrogenase (Mdh), *Mycobacterium smegmatis* lactate oxidase (Lox) and L-lactate dehydrogenase from *E. coli* (LctD). The positions of Ala-198 and Leu-230 are indicated.

consisted of 0.01 M HCl titrated against Tris solution to pH 7.5, and adjusted to 1.0 mol/l by addition of NaCl.

Rates of reduction of 1 mM ferricyanide (potassium salt; BDH Chemicals) by enzyme/substrate combinations were measured on a Beckman DU52 spectrophotometer as previously described [13].

Substrate solutions were prepared in Tris/HCl buffer, and titrated with 0.25 M NaOH to pH 7.5 before use; they contained glycolic acid (Aldrich), L-lactate (lithium salt; Sigma) and the following enantiomerically pure long-chain 2-hydroxy acids (produced by Oxford Asymmetry Ltd.), which correspond to the same stereochemistry as L-lactate: (S)-2-hydroxybutyric acid $\{[\alpha]_D^{25} 12.7^\circ$ (c 1.1 in chloroform)); (S)-2-hydroxyvaleric acid $\{[\alpha]_D^{25} -1.9^\circ$ (c 1.0 in ethanol)); (S)-2-hydroxyhexanoic acid $\{[\alpha]_D^{25} -1.9^\circ$ (c 1.0 in ethanol)); (S)-2-hydroxyoctanoic acid $\{[\alpha]_D^{25} 1.0^\circ$ (c 1.0 in ethanol)).

Kinetic isotope effects (KIEs) were measured as previously reported [13], using L-[2- ^3H]lactate prepared as described in [14].

RESULTS

Steady-state kinetic parameters

Table 1 presents the data collected from steady-state kinetic analyses of wild-type and mutant flavocytochromes b_2 , with

Figure 2 A parallel-vision stereoscopic view of the flavocytochrome b_2 active site

Pyruvate is shown in the centre of the diagram with residues Leu-230 and Ala-198 bottom right and bottom left respectively. Arg-376 lies to the left of pyruvate, and His-373 at the top. FMN is shown in a parallel plane directly behind the substrate.

Table 1 Steady-state parameters for wild-type and mutant flavocytochromes b_2 with substrates of different chain length

All experiments were carried out at 25.0 ± 0.1 °C in Tris/HCl buffer, pH 7.5 (/0.10). Values for k_{cat} represent the rates of reduction, at saturating substrate concentration, of 1 mM ferricyanide in electrons transferred /s per molecule of enzyme. As each substrate is a 2-electron donor, k_{cat} values should be halved to give rates in terms of substrate molecules consumed. K_m values are expressed in terms of mM substrate concentration. All substrates used are the simple straight-chained 2-hydroxy acids with two to eight carbon atoms. Errors quoted represent standard deviations from a non-linear least-squares fit.

Substrate	Chain length	Wild-type		A198G		L230A		A198G/L230A	
		k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
Glycollate	2	$7 \pm 1^*$	$0.34 \pm 0.05^*$	3 ± 1	2 ± 1	3 ± 1	3 ± 1	—	—
L-Lactate	3	$400 \pm 10^\dagger$	$0.49 \pm 0.05^\dagger$	185 ± 5	4.1 ± 0.4	30 ± 3	6.1 ± 0.2	41 ± 2	38 ± 4
(S)-2-Hydroxybutyrate	4	82 ± 7	0.59 ± 0.04	34 ± 1	2.4 ± 0.3	18 ± 2	0.83 ± 0.05	13 ± 2	3.6 ± 0.5
(S)-2-Hydroxyvalerate	5	13 ± 1	0.22 ± 0.02	4.0 ± 0.2	0.85 ± 0.08	19 ± 2	0.24 ± 0.02	21 ± 3	2.1 ± 0.2
(S)-2-Hydroxyhexanoate	6	19 ± 1	0.11 ± 0.01	5.3 ± 0.5	0.46 ± 0.05	16 ± 2	0.16 ± 0.01	25 ± 1	1.5 ± 0.1
(S)-2-Hydroxyoctanoate	8	45 ± 5	0.11 ± 0.01	14 ± 1	0.60 ± 0.03	52 ± 5	0.23 ± 0.01	68 ± 3	1.9 ± 0.1

* Taken from [16].

† Taken from [13].

Table 2 [^2H]Lactate KIEs

All experiments were carried out at 25.0 ± 0.1 °C in Tris/HCl buffer, pH 7.5 (/0.10). Ferricyanide was used as the electron acceptor, at a concentration of 1 mM. Values of k_{cat} and K_m are expressed as in Table 1.

Substrate	Wild-type*		A198G		L230A	
	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
L-[2- ^2H]Lactate	400 ± 10	0.49 ± 0.05	185 ± 5	4.1 ± 0.4	30 ± 3	6.1 ± 0.2
L-[2- ^2H]Lactate	86 ± 1	0.76 ± 0.06	51 ± 10	6.3 ± 1.0	5.1 ± 0.5	5.2 ± 0.3
KIE	4.7 ± 0.4		3.6 ± 1.0		5.9 ± 1.3	

* Taken from [13].

substrates of increasing chain length. All substrates used were enantiomerically pure *S*-isomers, to avoid complications caused by *R*-isomer inhibition, as observed for D-lactate [15].

The first point to note is that for a given enzyme, K_m values are lower with long-chain 2-hydroxy acids than with short. As it has been demonstrated that the reaction closely follows Michaelis-Menten kinetics (although substrate inhibition is observed at high concentration) [17], in which K_m is equated to $1/K_d$, K_m values can be used as an indication of the enzyme's relative substrate-binding affinity. Hence the apparent relationship between long chains and stronger binding can be rationalized in terms of the increase in size of the hydrophobic 'tail' on the substrate molecules. Such large non-polar substituents, repelled by solvent molecules, would clearly bind more effectively within the protein interior. Despite this trend, a K_m of 0.49 mM is obtained for wild-type flavocytochrome b_2 with L-lactate, which is slightly lower than that obtained with the longer (S)-2-hydroxybutyrate. Furthermore the k_{cat} of 400 s^{-1} is significantly higher than with all other substrates. On this evidence L-lactate is the optimum substrate for the enzyme.

For the A198G mutant enzyme the highest k_{cat} is also observed with L-lactate, although it is less than half the value observed with wild-type. Similarly k_{cat} values obtained with the other substrates are two or three times lower than the corresponding wild-type values. In addition to this universal decrease in k_{cat} , there is an increase in K_m across the substrate range; however, the increase for L-lactate is slightly out of proportion, resulting in this being the weakest binding of all the substrates. Since the

changes in k_{cat} and K_m which arise from the A198G mutation are of a universal nature, the overall effect on substrate specificity is modest.

The K_m values obtained for the L230A enzyme with long-chain 2-hydroxy acids are similar to the corresponding wild-type values, whereas those for short-chain substrates (glycollate and L-lactate) are 10-fold higher. Therefore, compared with wild-type, this represents a large decrease in the binding affinity of the L230A enzyme for the smaller substrates. In addition to this, the k_{cat} values obtained for L230A with small substrates [glycollate, L-lactate and (S)-2-hydroxybutyrate] are much lower than for the wild-type enzyme, k_{cat} for L-lactate showing a decrease of more than 10-fold as a consequence of the L230A mutation. As k_{cat} for L-lactate with the L230A mutant is now lower than for (S)-2-hydroxyoctanoate, and its K_m considerably higher, L-lactate can no longer be considered the primary substrate for this mutant. Other more subtle kinetic changes caused by the L230A mutation include an increase in k_{cat} for (S)-2-hydroxyvalerate, and increases in K_m values for both (S)-2-hydroxyhexanoate and (S)-2-hydroxyoctanoate. The double mutation A198G/L230A causes the collective swings in kinetic performance demonstrated by the single mutants. As such, k_{cat} values obtained for this enzyme are very similar to the values observed with the L230A mutant, throughout the substrate range. However, the K_m values, which show a universal increase, are more reminiscent of values recorded for the A198G enzyme, showing further disruption of substrate binding. The loss of all significant activity in the case of glycollate is presumably an extension of this phenomenon.

KIEs

Abstraction of the α -H from L-lactate is known to be the major rate-limiting step in the dehydrogenation pathway of flavocytochrome b_2 . This is demonstrated by the large KIE of 4.7 [13] observed during steady-state reduction of ferricyanide with L-[2- 3 H]lactate as substrate. Table 2 presents similar parameters obtained for the A198G and L230A mutant enzymes. As the KIEs remain large for the mutants, it is most likely that the rate-determining step and transition state are identical in both mutant and wild-type enzymes.

DISCUSSION

Flavocytochrome b_2 from *S. cerevisiae* is physiologically an L-lactate dehydrogenase, but it is also able to utilize other 2-hydroxy acids as substrates. The high-resolution crystal structure of the enzyme reveals a product molecule, pyruvate, bound in the active site by Arg-376 and Tyr-143 at the carboxylate end and

His-373 and Tyr-254 at the carbonyl. These residues are believed to be responsible for the group-specific nature of the enzyme. However, it is clear that they cannot be responsible for the ability of the enzyme to distinguish between L-lactate and other 2-hydroxy acids, i.e. its substrate specificity. The selectivity of wild-type flavocytochrome b_2 for L-lactate over 2-hydroxy acids of different chain length is illustrated by the substrate-specificity profiles in Figures 4(a) and 4(b), which show a conspicuously large peak in enzyme efficiency for chain length 3. The reason for this discrimination must arise from the extra stability gained by the Michaelis complex and transition state of the enzyme from interactions between the L-lactate/pyruvate methyl group and strategically positioned amino acid side chains. The underlying trend in the substrate-specificity profile is a general increase in enzyme efficiency with increasing chain length, which can be attributed to the greater binding affinity of longer, more hydrophobic substrates for the active site, as mentioned above. Despite this trend, L-lactate is a more efficient substrate than (S)-2-hydroxyoctanoate by a factor of 2.

The flavin-binding domain of flavocytochrome b_2 is closely related to several other 2-hydroxy acid dehydrogenases with different substrate specificities. These include the glycollate oxidase from spinach [18], lactate oxidase from *Mycobacterium smegmatis* [19], lactate dehydrogenase from *E. coli* [20], mandelate dehydrogenase from *Pseudomonas putida* [21] and long-chain hydroxy acid dehydrogenase from rat kidney [22]. The catalytically important residues are well conserved throughout this family but we have sought to determine the structural basis for substrate selectivity. The crystal structure of *S. cerevisiae* flavocytochrome b_2 indicates that the side chains of Ala-198 and Leu-230 interact closely with the methyl group of pyruvate, the product of lactate oxidation, which is found at the active site of the enzyme. The amino acid sequences of flavocytochrome b_2 and its relatives are compared for this region of the polypeptide chain in Figure 3. Ala-198 is replaced by glycine in some members of the family, whereas the position of Leu-230 is occupied by the larger tryptophan side chain in glycollate oxidase and by the smaller alanine in mandelate dehydrogenase. It thus appears plausible that these residues play some role in substrate selection. In an attempt to demonstrate the importance of these residues in controlling the substrate specificity in this series of enzymes, three mutant flavocytochromes b_2 were constructed: A198G, L230A and the double mutant A198G/L230A. As such, in each mutant enzyme, bulk had been removed from the appropriate region of the active site, and we expected that this would swing the substrate specificity of the enzyme away from L-lactate and towards bulkier 2-hydroxy acids. A similar study by Wilks et al. [23] involved the NAD $^{+}$ -dependent L-lactate dehydrogenase from *Bacillus stearothermophilus*. The enzyme was modified to become a broad-specificity 2-hydroxy acid dehydrogenase by the removal of a large amount of steric bulk from the active site. Although the mechanism and direction of the enzyme-catalysed reaction are different in this case, the general principle is the same.

As a means of quantifying the effect of each mutation on substrate specificity, steady-state analyses were carried out on the wild-type and mutant enzymes for a range of 2-hydroxy acids of increasing chain length. The results have been used to build up substrate-specificity profiles for each enzyme, as represented in Figure 4, and in Table 3.

By comparing the relative rates of reaction of L-[2- 1 H]- and L-[2- 2 H]-lactate with the A198G and L230A enzymes, KIEs were calculated. The large primary effects observed served to confirm that α -H abstraction is the rate-determining step in both cases. As the same result has been reported for the wild-type enzyme,

Figure 4 Substrate-specificity profiles of wild-type and mutant enzymes

(a) Comparison of wild-type (■) with A198G (▨); (b) comparison of wild-type (■) with L230A (▤); (c) comparison of L230A (▤) with A198G/L230A (▦). Values for k_{cat}/K_m are taken from Table 3 and are presented in $\text{mM}^{-1} \cdot \text{s}^{-1}$ on a log scale. Substrate chain lengths (2–8) correspond to the (S)-2-hydroxy acids in Table 1.

Table 3 Second-order rate constants for wild-type and mutant flavocytochromes b_2 with substrates of different chain length

All values are calculated from the data presented in Table 1.

Substrate	Chain length	Wild-type	A198G	L230A	A198G/L230A
		$k_{\text{cat.}}/K_m$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	$k_{\text{cat.}}/K_m$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	$k_{\text{cat.}}/K_m$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	$k_{\text{cat.}}/K_m$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Glycollate	2	$20 \pm 3^*$	1.5 ± 0.9	1 ± 0.5	—
L-Lactate	3	$810 \pm 90^\dagger$	45 ± 5	4.9 ± 0.5	1.1 ± 0.1
(S)-2-Hydroxybutyrate	4	140 ± 15	14 ± 2	22 ± 3	3.6 ± 0.2
(S)-2-Hydroxyvalerate	5	59 ± 7	4.7 ± 0.5	79 ± 10	10 ± 2
(S)-2-Hydroxyhexanoate	6	173 ± 18	11.5 ± 1.5	100 ± 14	17 ± 1
(S)-2-Hydroxyoctanoate	8	410 ± 60	23 ± 2	226 ± 24	36 ± 3

* Taken from [16].

† Taken from [13].

it is safe to assume that the mechanism followed and transition state passed through are consistent in all three enzymes. This allows the kinetic data to be compared and interpreted with respect to the same fundamental model.

For example, the A198G mutation causes increases in K_m for all the substrates, indicating that substrate binding has been weakened, and the Michaelis complex destabilized. Decreases in $k_{\text{cat.}}$ throughout the substrate range suggest that the transition state is also destabilized, the effect being unspecific in both cases. A possible explanation for this is that Ala-198 interacts with the β -CH₂ (possessed by all substrates except glycollate), in an unspecific way, its removal causing universal disruption. This would be the case if Ala-198 could interact without causing a steric obstruction to the long-chain substrates. Figure 4(a) shows the substrate-specificity profile of A198G in comparison with the wild-type profile, enzyme efficiency ($k_{\text{cat.}}/K_m$) being plotted against substrate chain length. It is clear that wild-type flavocytochrome b_2 is a far more efficient 2-hydroxy acid dehydrogenase than A198G, by an order of magnitude, but that the substrate specificity is largely unaffected by the mutation. Although Ala-198 clearly influences catalysis, it is not responsible for the selection of L-lactate over the other substances studied.

L230A on the other hand has a substrate-specificity profile which differs dramatically from the wild-type profile, as illustrated in Figure 4(b). The catalytic efficiency ($k_{\text{cat.}}/K_m$) of the mutant enzyme increases progressively with substrate chain length, whereas the wild-type profile includes a large peak in the L-lactate position (chain length 3). Significantly, this peak has been all but completely eradicated in the L230A profile. It is clear therefore that Leu-230 is critically involved in the substrate chain-length selection process, influencing both $k_{\text{cat.}}$ and K_m for the dehydrogenation reaction such as to optimize the enzyme's efficiency with L-lactate. Replacement of this residue by alanine removes the favourable interaction between the hydrocarbon chain of Leu-230 and the methyl group of L-lactate, with the possible consequence of forcing water molecules to occupy this hydrophobic pocket. However, the longer 2-hydroxy acids have hydrocarbon chains that would be able to contact the mutated Ala-230 residue, causing an interaction similar to the alkyl-alkyl interaction believed to occur with Leu-230. The observation that (S)-2-hydroxyvalerate (chain length 5) has an improved catalytic efficiency with the mutant suggests that this substrate is of the appropriate length to occupy the pocket, thus avoiding the possibility of steric crowding.

The result of the L230A mutation is therefore an enzyme that is best described as a long-chain 2-hydroxy acid dehydrogenase,

Figure 5 Branching at the β position of the substrate chain impedes the catalytic dehydrogenation reaction

rather than an L-lactate dehydrogenase. Such is the degree of L-lactate deselection by the mutant that it is now 40 times more efficient with (S)-2-hydroxyoctanoate than with L-lactate. In comparison, wild-type flavocytochrome b_2 is twice as efficient with L-lactate as with (S)-2-hydroxyoctanoate. Therefore the L230A mutation has caused an 80-fold swing in selectivity.

Progression to the double mutation A198G/L230A causes even larger increases in the K_m values, signifying a large disruption to the substrate-binding properties of the enzyme. However, the $k_{\text{cat.}}$ values are similar to those recorded with the L230A mutant enzyme, rather than those obtained with the A198G mutant, which are somewhat lower. This suggests that the L230A mutation in some way compensates for the A198G mutation with respect to $k_{\text{cat.}}$. The substrate-specificity profile of the A198G/L230A mutant (Figure 4c) therefore resembles the L230A profile in shape, but poor substrate-binding properties cause the enzyme to be far less efficient in every case.

An additional observation was that none of the mutants studied showed any activity with (S)-2-hydroxyisovalerate (Figure 5), whereas the activity with S-2-hydroxyisohexanoate was similar to that observed with (S)-2-hydroxyhexanoate. It appears therefore that, whereas branching at C-4 causes no ill effect, branching at C-3 invokes steric interference, presumably from a different active-site residue, which exerts its own influence on substrate specificity. One possibility is Tyr-254, the aromatic ring of which is seen to be within contact distance of the pyruvate methyl group in the crystal structure. Mutation of this residue will be the subject of further investigation.

In conclusion, the substrate-specificity studies that we have carried out on wild-type flavocytochrome b_2 and the A198G, L230A and A198G/L230A mutant enzymes have shown that Leu-230 is a key residue responsible for selecting L-lactate as the primary substrate for the enzyme in preference to other 2-hydroxy acids. Further, in generating the L230A mutant we have

demonstrated the possibilities for successfully redesigning the enzyme to select other 2-hydroxy acids in preference to the natural substrate. This mutant enzyme is more efficient at utilizing long-chain 2-hydroxy acids, such as (*S*)-2-hydroxyoctanoate, by a factor of 40.

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