Re-design of *Saccharomyces cerevisiae* flavocytochrome b_2 : introduction of L-mandelate dehydrogenase activity

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Flavocytochrome b_2 from *Saccharomyces cerevisiae* is an L-lactate dehydrogenase which exhibits only barely detectable activity levels towards another 2-hydroxyacid, L-mandelate. Using protein engineering methods we have altered the active site of flavocytochrome b_2 and successfully introduced substantial mandelate dehydrogenase activity into the enzyme. Changes to Ala-198 and Leu-230 have significant effects on the ability of the enzyme to utilize L-mandelate as a substrate. The double mutation of Ala-198 → Gly and Leu-230 → Ala results in an enzyme with a k_{cat} value (25 °C) with L-mandelate of 8.5 s⁻¹, which represents an increase of greater than 400-fold over the wild-type enzyme. Perhaps more significantly, the mutant enzyme has a catalytic efficiency (as judged by k_{cat}/K_m values) that is 6-fold higher with L-mandelate than it is with L-lactate. Closer examination of the X-ray structure of *S. cerevisiae* flavocytochrome b_2 led us to

conclude that one of the haem propionate groups might interfere with the binding of L-mandelate at the active site of the enzyme. To test this idea, the activity with L-mandelate of the independently expressed flavodehydrogenase domain (FDH), was examined and found to be higher than that seen with the wild-type enzyme. In addition, the double mutation of Ala-198 → Gly and Leu-230 → Ala introduced into FDH produced the greatest mandelate dehydrogenase activity increase, with a k_{cat} value more than 700-fold greater than that seen with the wild-type holoenzyme. In addition, the enzyme efficiency (k_{cat}/K_m) of this mutant enzyme was more than 20-fold greater with L-mandelate than with L-lactate. We have therefore succeeded in constructing an enzyme which is now a better mandelate dehydrogenase than a lactate dehydrogenase.

INTRODUCTION

The flavocytochrome b_2 from *Saccharomyces cerevisiae* is a homotetrameric enzyme that catalyses the oxidation of lactate to pyruvate in the mitochondrial intermembrane space. The three-dimensional structure of the enzyme [1] shows that each subunit consists of two domains: a flavodehydrogenase domain, containing FMN, and a cytochrome domain, containing protohaem IX. These two domains are joined by a polypeptide linker which is believed to function as a hinge [2]. The catalytic cycle of the enzyme involves the two-electron reduction of the flavin by L-lactate [3]. The flavin then passes electrons singly to the haem [4], from which they are passed to the physiological acceptor cytochrome *c*. The key residues involved in the dehydrogenation of L-lactate have been identified in the crystal structure [1], and in many cases their roles have been confirmed by site-directed mutagenesis [5–7]. A carbanion mechanism has been proposed for the oxidation of L-lactate [8], although a hydride transfer from L-lactate to flavin N-5 is equally plausible, as has been suggested in the case of D-amino acid oxidase [9].

The extent of the substrate specificity of *S. cerevisiae* flavocytochrome b_2 is confined to a range of (S)-2-hydroxy acids [10]. This substrate specificity has been strategically altered by site-directed mutagenesis to enable the enzyme to work more efficiently with long-chain 2-hydroxyacids [11]. However, one 2-hydroxyacid with which *S. cerevisiae* flavocytochrome b_2 shows very little activity (at the limits of detection) is L-mandelate. This is somewhat surprising when one considers the extensive similarity between *S. cerevisiae* flavocytochrome b_2 and the L-

mandelate dehydrogenase from *Rhodotorula graminis* reported in the preceding paper [12]. Consideration of the three-dimensional structure of *S. cerevisiae* flavocytochrome b_2 has led to the identification of several residues at the active site which might be responsible for the discrimination of the enzyme against L-mandelate [13]. These include Ala-198, Leu-230 and Ile-326. The present study presents the results of a protein engineering programme with the aim of converting *S. cerevisiae* flavocytochrome b_2 into an L-mandelate dehydrogenase.

MATERIALS AND METHODS

DNA manipulation, strains, media and growth

Site-directed mutagenesis was performed by the Kunkel method of non-phenotypic selection [14] using the oligonucleotides CTGCTAACAGGTTTGTGTAAC, GATATCTACTGCTGCTTCATGT and GATATCTACTGGTGCTTCATGT to construct the mutant enzymes Ala-198 → Gly, Leu-230 → Ala and Leu-230 → Gly respectively. In addition, the double mutant Ala-198 → Gly/Leu-230 → Ala was constructed in the intact enzyme and also in its independently expressed flavin domain, by recombining the appropriate restriction cleavage fragments from singly modified plasmids. Standard methods for growth of *Escherichia coli*, plasmid purification, DNA manipulation and transformation were performed as described in Sambrook et al. [15]. *E. coli* strain TG1 was used for expression of wild-type and mutant flavocytochromes b_2 .

Abbreviation used: FDH, flavodehydrogenase domain.

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Enzymes

Wild-type and mutant flavocytochromes b_2 expressed in *E. coli* were isolated from cells stored in liquid nitrogen. The purification procedure was essentially as described previously [16]. Short-term storage (days) of purified enzyme was as 70% ammonium sulphate precipitates at 4 °C under nitrogen in the reduced state. For long-term storage (weeks, months) the enzyme was oxidized, separated into 100 μ l aliquots, snap-frozen and stored in liquid nitrogen. Under these conditions the enzyme retained full activity indefinitely. Flavocytochrome b_2 concentrations were calculated using previously published absorption coefficients [17].

Kinetic analysis

All experiments were carried out at 25 ± 0.1 °C in 10 mM Tris/HCl at pH 7.5, $I_{0.10}$. The buffer was 10 mM in HCl, adjusted to pH 7.5 with Tris and corrected to $I_{0.10}$ by addition of NaCl. Ferricyanide (potassium salt; BDH) at a concentration of 1 mM (for the intact enzyme) and 6 mM (for the flavodehydrogenase domain) was used as the electron acceptor in all steady-state experiments, as previously described [5]. Steady-state measurements of the enzymic oxidation of both L-mandelate and L-lactate were performed on a Shimadzu UV2101PC spectrophotometer. K_m and k_{cat} values were determined using non-linear regression analysis.

RESULTS AND DISCUSSION

The X-ray crystal structure of *S. cerevisiae* flavocytochrome b_2 [1] has allowed the active site for 2-hydroxyacid dehydrogenation to be identified. In fact, in one of the two crystallographically distinguishable subunits in the asymmetric unit, a molecule of pyruvate (the product of the reaction) can be found. A representation of this is shown in Figure 1 where it can be seen that the methyl group of pyruvate makes contact with the hydrophobic side-chains of Ala-198 and Leu-230, while being a little more distant from Ile-326. It would appear likely then that these interactions are responsible for the selection of L-lactate by the enzyme in preference to other 2-hydroxyacids.

S. cerevisiae flavocytochrome b_2 is related both structurally and by sequence to a number of other FMN-containing 2-hydroxyacid dehydrogenases/oxidases, including spinach glycolate oxidase [18] and *R. graminis* L-mandelate dehydrogenase [12]. However, these enzymes exhibit a kinetic preference for alternative substrates. The short sequence comparison in the preceding paper by Illias et al. (Figure 3 of that paper) [12] indicates that residues Ala-198 and Leu-230, highlighted in Figure 1, are not well conserved in these enzymes, adding support to the idea that they are important in substrate selection.

The role of these amino acids in substrate specificity has been examined in previous studies [11,19] in which the residues were all replaced by smaller variants in an attempt to increase the size of the substrate-binding cavity. The activities of the mutant enzymes were examined with a range of straight-chain 2-hydroxyacids. Substitution of Leu-230 by Ala converted the enzyme into a non-specific long-chain 2-hydroxyacid dehydrogenase [11]. Substitution of Ile-326 by Ala, on the other hand, generated a specific L-2-hydroxyoctanoate dehydrogenase [19]. The influence of Ile-326 is likely to be complex, since it lies in direct contact with Leu-230 and probably contacts the alkyl substituents of the larger substrates.

The activity of *S. cerevisiae* flavocytochrome b_2 towards L-mandelate is near the limits of reliable measurement, with a k_{cat} value (25 °C) of only 0.02 s^{-1} (compared with a value of 400 s^{-1} seen with L-lactate). This very low rate makes an accurate K_m

Figure 1 Active site of *S. cerevisiae* flavocytochrome b_2

Hydrophobic interactions are indicated between the methyl group of pyruvate and various alkyl amino acid side-chains.

value virtually impossible to determine, but it is clear that the catalytic efficiency of the enzyme with L-mandelate is very low indeed. In order to engineer a shift in activity towards L-mandelate and away from L-lactate, the following mutants were generated and tested: Ala-198 $\rightarrow$ Gly, Leu-230 $\rightarrow$ Ala, Leu-230 $\rightarrow$ Gly, Ile-326 $\rightarrow$ Ala and the double mutation Ala-198 $\rightarrow$ Gly/Leu-230 $\rightarrow$ Ala. An attempt was also made to generate the Ala-198 $\rightarrow$ Gly/Leu-230 $\rightarrow$ Gly double mutant, but all efforts to obtain active enzyme failed. It might be that this double mutation makes such a large cavity at the active site that the enzyme is no longer stable and the active site may collapse. Steady-state kinetic parameters for all of these mutant enzymes, with both L-mandelate and L-lactate as substrates, are listed in Table 1. An examination of Table 1 indicates that, although the various mutations have effects on both k_{cat} and K_m values, the more profound effects are on k_{cat} , i.e. the rate of turnover with L-mandelate. The shift in catalytic efficiency (k_{cat}/K_m) for the various mutant enzymes with L-mandelate and L-lactate as substrates is clearly seen in the specificity profile shown in Figure 2.

The Ile-326 $\rightarrow$ Ala mutation makes virtually no improvement to the activity of the enzyme with L-mandelate, but it does cause a dramatic fall in the k_{cat} value seen with L-lactate (from 400 s^{-1} down to 11 s^{-1}). The Ala-198 $\rightarrow$ Gly mutation on the other hand results in a 20-fold increase in the k_{cat} values with L-mandelate, which is a significant improvement. Mutations at position 230 are rather more interesting. Changing Leu-230 to Ala results in only a 5-fold increase in the k_{cat} value with L-mandelate. However, changing this residue to a glycine, the equivalent residue at this position in *R. graminis* L-mandelate dehydrogenase, causes a much larger 40-fold increase in the k_{cat} value (Table 1). This highlights the importance of Leu-230 in substrate selectivity. The biggest increase in activity with the holo-enzyme towards L-

Table 1 Steady-state parameters for wild-type and mutant flavocytochromes b_2 with L-mandelate and L-lactate

All experiments were carried out at 25 ± 0.1 °C in 10 mM Tris/HCl at pH 7.5 (/0.10), with ferricyanide (1 mM), as electron acceptor. Values for k_{cat} are expressed as electrons transferred per second per molecule of enzyme. K_m values are expressed in terms of mM of substrate. All errors quoted represent standard deviations from a non-linear least-squares fit. Activities were so low in the case of L-mandelate with wild-type flavocytochrome b_2 that it was impossible to determine a meaningful K_m value. ND, not determined.

Enzyme	L-Mandelate			L-Lactate		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Wild-type	$0.02 (\pm 0.01)$	ND	ND	$400 (\pm 10)$	$0.49 (\pm 0.05)$	$816 (\pm 116)$
Ile-326 → Ala	$0.04 (\pm 0.02)$	ND	ND	$11 (\pm 1)$	$0.76 (\pm 0.07)$	$14 (\pm 3)$
Ala-198 → Gly	$0.40 (\pm 0.05)$	$1.00 (\pm 0.20)$	$0.40 (\pm 0.16)$	$185 (\pm 5)$	$4.10 (\pm 0.40)$	$45 (\pm 6)$
Leu-230 → Ala	$0.11 (\pm 0.05)$	$0.16 (\pm 0.01)$	$0.69 (\pm 0.38)$	$30 (\pm 3)$	$6.10 (\pm 0.20)$	$4.9 (\pm 0.7)$
Leu-230 → Gly	$0.80 (\pm 0.20)$	$0.70 (\pm 0.10)$	$1.14 (\pm 0.53)$	$100 (\pm 5)$	$1.90 (\pm 0.30)$	$53 (\pm 9.8)$
Ala-198-Gly/Leu-230 → Gly	$8.50 (\pm 0.50)$	$1.36 (\pm 0.10)$	$6.25 (\pm 0.89)$	$41 (\pm 2)$	$38 (\pm 4)$	$1.08 (\pm 0.15)$

Figure 2 Substrate specificity profile for wild-type and mutant flavocytochromes b_2

Comparison of the various enzymes with L-mandelate and L-lactate as substrates. The star symbols above the WT (wild type) and I326A (Ile-326 → Ala) indicate values at the limits of accurate determination. The axis for k_{cat}/K_m is represented on a logarithmic scale.

mandelate, however, is seen with the double mutation of Ala-198 → Gly plus Leu-230 → Ala. This double mutation results in an enzyme with a k_{cat} value with L-mandelate of 8.5 s^{-1} , which represents an increase of greater than 400-fold over the wild-type

enzyme. Perhaps more significantly, the mutant enzyme has a catalytic efficiency (as judged by k_{cat}/K_m values) which is 6-fold higher with L-mandelate than it is with L-lactate (Table 1).

Further examination of the X-ray structure of *S. cerevisiae* flavocytochrome b_2 led us to conclude that one of the haem propionate groups might interfere with the binding of L-mandelate at the active site of the enzyme. The flavin and haem groups are actually quite close together, with one of the haem propionate oxygen atoms coming within ~ 6 Å of the flavin N-5 [1]. To test whether or not this propionate group hindered the correct binding of L-mandelate in either wild-type or mutant flavocytochromes b_2 we examined the activity of the independently expressed flavodehydrogenase domain (FDH) towards L-mandelate. The results (Table 2) indicated that the propionate might indeed be having some effect since the k_{cat} value for FDH with L-mandelate was 0.1 s^{-1} , compared with 0.02 s^{-1} seen with the holoenzyme. To examine this effect further we introduced the Ala-198 → G/Leu-230 → Ala double mutation into FDH, which resulted in an enzyme with the highest mandelate dehydrogenase activity yet seen (Table 2). In fact the FDH Ala-98 → Gly/Leu-230 → Ala mutant enzyme has a k_{cat} value with L-mandelate more than 700-fold greater than that seen with the wild-type holoenzyme. In addition, the enzyme efficiency (k_{cat}/K_m) of FDH Ala-198 → Gly/Leu-230 → Ala is more than 20-fold greater with L-mandelate than it is with L-lactate (Figure 2). We have therefore succeeded in constructing an enzyme which is now a better mandelate dehydrogenase than a lactate dehydrogenase.

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Table 2 Steady-state parameters for the flavocytochrome b_2 FDH and the FDH Ala-198 → Gly/Leu-230 → Ala mutant enzyme with L-mandelate and L-lactate

All experiments were carried out at 25 ± 0.1 °C in 10 mM Tris/HCl at pH 7.5 (/0.10), with ferricyanide (6 mM) as electron acceptor. Values for k_{cat} are expressed as electrons transferred per second per molecule of enzyme. K_m values are expressed in terms of mM substrate. All errors quoted represent standard deviations from a non-linear least-squares fit.

	L-Mandelate		L-Lactate	
	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
FDH	$0.10 (\pm 0.05)$	$0.60 (\pm 0.10)$	$273 (\pm 6)$	$0.22 (\pm 0.05)$
FDH Ala-198 → Gly/Leu-230 → Ala	$15.5 (\pm 1.0)$	$0.44 (\pm 0.10)$	$32 (\pm 2)$	$20 (\pm 4)$

REFERENCES

- 1 Xia, Z.-X. and Mathews, F. S. (1990) *J. Mol. Biol.* **212**, 837–863
- 2 Sharp, R. E., Chapman, S. K. and Reid, G. A. (1996) *Biochemistry* **35**, 891–899
- 3 Daff, S., Ingledew, W. J., Reid, G. A. and Chapman, S. K. (1996) *Biochemistry* **35**, 6345–6350
- 4 Chapman, S. K., Reid, G. A., Daff, S., Sharp, R. E., White, P., Manson, F. D. C. and Lederer, F. (1994) *Biochem. Soc. Trans.* **22**, 713–718
- 5 Miles, C. S., Rouviere-Fourmy, N., Lederer, F., Mathews, F. S., Reid, G. A., Black, M. T. and Chapman, S. K. (1992) *Biochem. J.* **285**, 187–192
- 6 Dubois, J., Chapman, S. K., Mathews, F. S., Reid, G. A. and Lederer, F. (1990) *Biochemistry* **29**, 6393–6400
- 7 Gaume, B., Sharp, R. E., Manson, F. D. C., Chapman, S. K., Reid, G. A. and Lederer, F. (1995) *Biochimie* **77**, 621–630
- 8 Lederer, F. (1991) in *Chemistry and Biochemistry of Flavoenzymes*, vol. 2 (Müller, F., ed.), pp. 153–242, CRC Press, Boca Raton, FL
- 9 Mattevi, A., Vanoni, M. A. and Curti, B. (1997) *Curr. Opin. Struct. Biol.* **7**, 804–810
- 10 Chapman, S. K., White, S. A. and Reid, G. A. (1991) *Adv. Inorg. Chem.* **36**, 257–301
- 11 Daff, S., Manson, F. D. C., Reid, G. A. and Chapman, S. K. (1994) *Biochem. J.* **301**, 829–834
- 12 Ilias, R. M., Sinclair, R., Robertson, D., Neu, A., Chapman, S. K. and Reid, G. A. (1998) *Biochem. J.* **333**, 107–115
- 13 Sinclair, R., Reid, G. A., Daff, S. and Chapman, S. K. (1997) in *Flavins and Flavoproteins 1996* (Stevenson, K. J., Massey, V. and Williams, C. H., eds.), pp. 579–582, University of Calgary Press, Calgary
- 14 Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488–492
- 15 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd. edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 16 Black, M. T., White, S. A., Reid, G. A. and Chapman, S. K. (1989) *Biochem. J.* **258**, 255–259
- 17 Pajot, P. and Groudinsky, O. (1970) *Eur. J. Biochem.* **12**, 158–164
- 18 Lindqvist, Y., Brändén, C.-I., Mathews, F. S. and Lederer, F. (1991) *J. Biol. Chem.* **266**, 3198–3207
- 19 Daff, S., Manson, F. D. C., Reid, G. A. and Chapman, S. K. (1994) *Biochem. Soc. Trans.* **22**, 282

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