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RATIONAL CONSTRUCTION OF A 2-HYDROXYACID DEHYDROGENASE WITH NEW SUBSTRATE SPECIFICITY

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Using site-directed mutagenesis on the lactate dehydrogenase gene from *Bacillus stearothermophilus*, three amino acid substitutions have been made at sites in the enzyme which we suggest in part determine specificity toward different hydroxyacids (R-CHOH-COOH). To change the preferred substrates from the pyruvate/lactate pair (R = $-CH_3$) to the oxaloacetate/malate pair (R = $-CH_2-COO^-$), the volume of the active site was increased (thr 246 -> gly), an acid was neutralized (asp-197 -> asn) and a base was introduced (gln-102 -> arg). The wild type enzyme has a catalytic specificity for pyruvate over oxaloacetate of 1000 whereas the triple mutant has a specificity for oxaloacetate over pyruvate of 500.

ABBREVIATIONS: LDH, lactate dehydrogenase; FBP, fructose-1,6-bisphosphate.

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Despite the severity and extent of these active site alterations, the malate dehydrogenase so produced retains a reasonably fast catalytic rate constant (20 s^{-1} for oxaloacetate reduction) and is still allosterically controlled by fructose-1,6-bisphosphate.

Evolved proteins are not only extremely effective catalysts for chemical conversions, as defined by the rate enhancements they achieve, they are also selective. Given a range of substrates with roughly the same potential to undergo a given chemical reaction, an enzyme will usually discriminate between the substrate it has evolved to transform and the others. To alter or broaden the specificity of a given enzyme it is necessary to retain the "central" chemistry while manipulating those parts of its structure responsible for selecting the natural substrate. If a substrate is small and there are few interactions with the enzyme, the catalytic and specificity sites are necessarily very close together. In these circumstances it is justifiable to argue that any engineering of enzyme structure giving rise to new specificities will be too close to the centre of the chemical conversion to avoid large penalties in rate enhancement.

To evaluate the validity of this argument, an attempt is described to convert an NADH-linked 2-hydroxyacid dehydrogenase which naturally selects pyruvate ($CH_3.CO.COO^-$) to an enzyme which selects oxaloacetate ($^-OOC.CH_2.CO.COO^-$). For this process of re-design two reactions are chosen which have similar redox potentials:

$$\text{Lactate} + NAD^+ = \text{Pyruvate} + NADH + H^+ \quad [K_{eq} = 2.9 \times 10^{-12} M]$$

$$\text{Malate} + NAD^+ = \text{Oxaloacetate} + NADH + H^+ \quad [K_{eq} = 5.0 \times 10^{-13} M]$$

The main elements used in the design of the new enzyme were the known three-dimensional structures of LDH (1-3), the homologous structure of pig cytoplasmic malate dehydrogenase (16-18), a detailed understanding of the LDH catalytic mechanism (4,5,8) and the computational chemistry framework COSMIC (19) run on the SERC Bristol Molecular Graphics Workstation.

Methods

Three site-directed mutations (6) were introduced into the LDH gene derived from *Bacillus stearothermophilus* (7) using methods described previously (8). To provide extra volume in the active site for the additional carboxylate group of malate/oxaloacetate, threonine-246 was replaced by glycine, to supply a carboxylate binding site, glutamine-102 was replaced by arginine and to remove potential charge repulsion, aspartate-197 was replaced by asparagine.

The mutant gene was expressed in *E. Coli* TG2 cells in the vector pKK223-3 (7) and the protein purified to homogeneity by chromatography on Blue Sepharose (F3GA) and DEAE CL-6B Sepharose as described for other mutant LDHs from this laboratory (9). The level of expression of this triple mutant was 20-25% of the soluble *E. Coli* protein.

Steady-state reaction rates were determined by following the change in A_{340} due to the formation or consumption of NADH. Transient kinetic measurements were made on a HI-TECH stopped-flow spectrometer (HI-TECH, Salisbury, U.K.) and deuterated NAD^2H (nicotinamide-4-^{2}H) for measuring primary isotope effects was made enzymatically by the method of Oppenheimer (10). The NADH-binding constant was determined from fluorescence polarization (11). Thermal stability measurements were made at pH 6 in 5 mM FBP at 90°C. The concentration of protein in the incubations was 2 μM (subunits) and samples were removed at intervals over 32 minutes and stored on ice prior to assay. The buffer for all experiments was 100 mM triethanolamine-HCl/NaOH. Oxaloacetate solutions were made up freshly to minimize decarboxylation to pyruvate and the initial burst of contaminant pyruvate reduction with wild-type LDH was ignored (12).

Results

The steady-state catalytic properties of the wild-type and the mutant enzymes, shown in table 1 , demonstrate that the mutations have reversed the lactate/malate specificity of the enzyme. The wild-type enzyme

Table 1. Steady-state properties of mutant and wild-type enzyme

		Pyruvate		Oxaloacetate	
		+FBP	-FBP	+FBP	-FBP
k_{cat}	WT	250	250	6	-
(s^{-1})	MUT	0.49	0.10	20	-
K_m	WT	0.06	3	1.5	-
(mM)	MUT	25	22	2.0	-
k_{cat}/K_m	WT	4200000	83000	4000	-
($M^{-1}s^{-1}$)	MUT	19.6	4.5	10000	440

		Lactate +FBP	Malate +FBP
k_{cat}/K_m	WT	450	0.002
($M^{-1}s^{-1}$)	MUT	0.1	2.3

The above constants were measured in steady-state conditions at pH 6 for pyruvate and oxaloacetate as substrates and at pH 8 with lactate and malate. For oxaloacetate assays the rate of pyruvate turnover from spontaneous decarboxylation was subtracted (12). FBP, where used, was present at a concentration of 5 mM. All assays were carried out at a saturating concentration of coenzyme (0.2 mM NADH and 10 mM NAD^+). The temperature was 25°C and protein concentrations were determined from A_{280}. WT= wild-type enzyme; MUT= Q102R,D197N,T246G triple mutant.

catalyses pyruvate reduction 1000-times more effectively than oxaloacetate reduction (based on the ratio of k_{cat}/K_m) whereas the mutant is 500-times more effective against oxaloacetate than against pyruvate. This change in specificity is mirrored in the other reaction direction where the wild type oxidizes lactate 23000-times more effectively than malate and the mutant oxidizes malate 23-times more effectively than lactate. Thus the mutations have changed the specificity in both reaction directions to the same extent, 500000-fold in favour of the malate/oxaloacetate redox pair.

The engineered enzyme retains allosteric control by fructose-1,6-bisphosphate (11); the stimulation in k_{cat}/K_m of the malate dehydrogenase

activity in the presence of 5mM FBP being 23-fold, compared with 50-fold in the parent enzyme. This result suggests that the major path of communication between effector and active site (2 nm) is not through residues 102, 197 or 246. Additional evidence that the mutations have not had widespread structural consequences in the protein molecule was obtained from the NADH binding properties and thermal stabilities of mutant and wild-type LDHs. The K_d for NADH at pH 6 is 2 μM for both enzymes and the $t_{1/2}$ values at 90°C in the presence of 5 mM FBP were 3.5 minutes for the wild-type and 3.0 minutes for the mutant (see Methods). The rate determining step in pyruvate reduction in wild-type *B. stearothermophilus* LDH (8), and in its eukaryotic counterparts (5), is a structural rearrangement of the ternary complex. Recent evidence identifies this as the closure of a loop of polypeptide (residues 98-110) over the active site (13). As all three of the mutations described here are either on this loop or in the region of the protein onto which it packs after closure, it is important to determine whether they have changed the nature of the rate-determining step in the engineered enzyme. To do this we compared the transient kinetics of substrate reduction by NADH and NAD^2H (the deuterium substitution is made at the transferred C4-hydrogen of the dihydronicotinamide ring). In these experiments the binary complex (E-NADH) is rapidly mixed with the substrate and the rate constant for product formation is measured from the first-order transient. This method of measurement is relatively insensitive to small quantities of inhibitory coenzyme breakdown product which may affect steady state experiments. The results are shown in fig.1 and reveal that for the reduction of both pyruvate and oxaloacetate by the mutant enzyme there is a significant deuterium isotope effect; the k_H/k_D ratios are 2.4 and 2.3 respectively. We conclude that, unlike the wild-type enzyme (k_H/k_D = 1.0 (8)), the rate of reaction in the mutant is limited by hydride-transfer (20 s^{-1} with oxaloacetate and 0.5 s^{-1} with pyruvate) and that loop-closure is faster than this (in the wild-type it closes at 250 s^{-1} (13)).

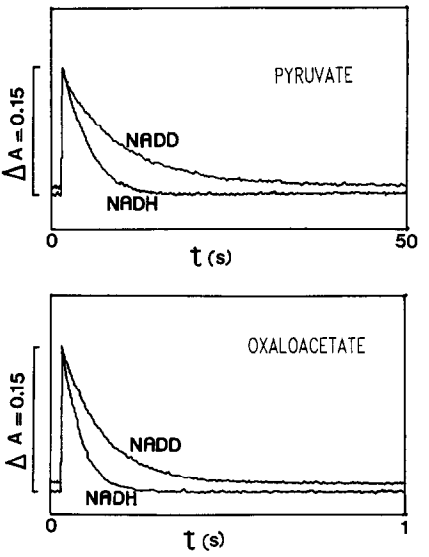

Figure 1. Deuterium isotope effects on the mutant enzyme in singleturnover conditions

A solution containing 25 μM enzyme sites, 25 μM NADH or NAD^2H and 5 mM FBP was rapidly mixed in a stopped-flow spectrometer with a solution of the substrate containing 5 mM FBP. The oxidation of the coenzyme was followed by measuring the disappearance of absorbance at 340 nm. With 40 mM pyruvate as substrate the first-order rate constants were 0.29 s^{-1} with NADH and 0.12 s^{-1} with NAD^2H, with 10 mM oxaloacetate these rates were 20 s^{-1} and 8.8 s^{-1}, respectively.

Discussion

Shown in fig.2a is a representation of the active site of lactate dehydrogenase in the E-NADH-pyruvate complex. In re-designing this site to select oxaloacetate, our concern was not to destroy the ability of the enzyme to facilitate the redox chemistry. Previous studies have revealed the catalytic importance of his-195 (proton donor/acceptor (4,5)), asp-168 (supports the protonated form of his-195 in the ternary complex (14,15)), ile-250 (supplies the hydrophobic environment for the dihydronicotinamide ring (15)), arg-109 (stabilizes negative charge on substrate C2-oxygen during transition state (8)) and arg-171 (provides a tight, bifurcated binding-interaction with the C1-carboxylate of the substrate (9)). To

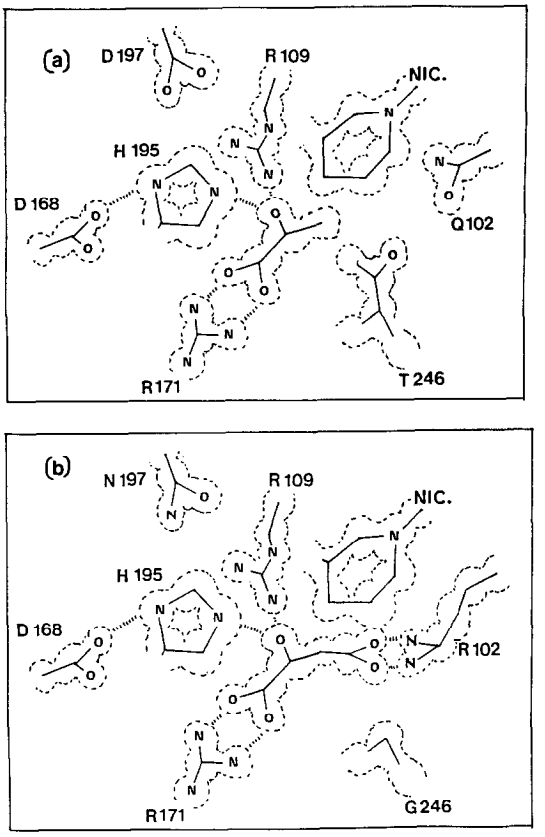

Figure 2. Schematic view of the active site modifications

The schematic view shown in (a) is a representation (generated by the algorithm FRODO) of the active site of LDH modelled by building the gene-derived amino acid sequence (7) of *B. stearothermophilus* LDH into the x-ray structure of the pig LDH NAD^+-S-lac complex (1) (all residues shown are invariant in the known LDH sequences). Diagram (b) shows the 3 changed amino acid residues and an oxaloacetate molecule at the original lactate binding-site.

accommodate the additional carboxylate group, whilst leaving these important elements intact, thr-246 which constrains the volume of the site has been changed to glycine, gln-102 has been replaced by arginine to provide a charge-pairing and asp-197 has been converted to the amide to avoid any negative charge repulsion with the second $-COO^-$ of the new substrate. The new active site is represented in fig 2b. The refined

model of cytosolic MDH is consistent with arginine-102 being important in malate/oxaloacetate binding (18).

Although these mutations have altered the active site structure in regions very close to the hydride-transfer centre, an extreme change in specificity is secured without sacrificing high catalytic rates. Work is underway to determine the individual contributions of these three mutations to specificity. This initial communication demonstrates, at least in the case of this large-volume and thermostable protein, that a catalyst for a defined biotransformation can be made by the rational engineering of an existing enzyme framework.

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