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REVIEW

Malate dehydrogenase: A model for structure, evolution, and catalysis

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Abstract

Malate dehydrogenases are widely distributed and alignment of the amino acid sequences show that the enzyme has diverged into 2 main phylogenetic groups. Multiple amino acid sequence alignments of malate dehydrogenases also show that there is a low degree of primary structural similarity, apart from in several positions crucial for nucleotide binding, catalysis, and the subunit interface. The 3-dimensional structures of several malate dehydrogenases are similar, despite their low amino acid sequence identity. The coenzyme specificity of malate dehydrogenase may be modulated by substitution of a single residue, as can the substrate specificity. The mechanism of catalysis of malate dehydrogenase is similar to that of lactate dehydrogenase, an enzyme with which it shares a similar 3-dimensional structure. Substitution of a single amino acid residue of a lactate dehydrogenase changes the enzyme specificity to that of a malate dehydrogenase, but a similar substitution in a malate dehydrogenase resulted in relaxation of the high degree of specificity for oxaloacetate. Knowledge of the 3-dimensional structures of malate and lactate dehydrogenases allows the redesign of enzymes by rational rather than random mutation and may have important commercial implications.

Keywords: malate dehydrogenase; molecular evolution; protein engineering

Many industrial processes involve chemical conversions of organic compounds where productivity is achieved by use of relatively nonspecific inorganic catalysts. Recent advances in the biological sciences have enhanced the potential for use of enzyme catalysts, which offer a number of advantages over conventional chemical catalysts, including high specificity for the reactants, improved reaction rates, and the use of mild operational conditions. There are already many examples of commercial applications where the properties of natural enzyme catalysts have been exploited to perform synthesis and degradation of compounds, as diagnostic reagents and in research applications. Enzymes isolated from natural sources have been optimized through evolution to perform a particular biological role and often have disadvantages for the design of specific chemical application protocols. Genetic manipulation via protein engineering allows the rational redesign of an enzyme primary sequence to change its chemical and physical properties. A lack of structural information for many enzymes means that the physiological effects of amino acid sequence alterations are difficult to predict. The enzyme malate dehydrogenase (MDH; EC 1.1.1.37) is a good candidate to test the efficiency of predictive amino acid sequence modification as it has been extensively characterized and many isozymes with different properties are available from a variety of sources.

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Distribution of MDHs

MDHs catalyze the interconversion of oxaloacetate and malate linked to the oxidation/reduction of dinucleotide coenzymes. Oxaloacetate plays a crucial role in many metabolic pathways including operation of the tricarboxylic acid cycle, glyoxylate bypass, amino acid synthesis, gluconeogenesis, maintenance of oxidation/reduction balance, and facilitation of the exchange of metabolites between cytoplasm and subcellular organelles. Consequently, MDH has been isolated from many diverse sources, including eubacteria, archaea, fungi, plants, and mammals, and from subcellular organelles such as mitochondria, chloroplasts, glyoxysomes, and peroxisomes.

MDHs are multimeric enzymes consisting of identical subunits usually organized as either dimers or tetramers with subunit molecular weights of between 30 and 35 kDa (Banaszak & Bradshaw, 1975; Sundaram et al., 1980). Each subunit functions independently in terms of catalysis (McEvily et al., 1985) with no evidence of cooperativity between catalytic sites. In general,

NAD^+-dependent MDHs are not subject to metabolic regulation, with the exception of the mitochondrial enzyme, which is dependent on allosteric control by citrate at 1 site per subunit (Gelpi et al., 1992). The $NADP^+$-dependent chloroplast MDHs are indirectly regulated by light. Active enzyme is produced after reduction of disulfide bridges in the N-terminus as a result of the electron transport system of photosynthesis (Gietl, 1992). Predictive modification of the enzyme was used to activate chloroplast MDH by substitution of both N- and C-terminal-exposed cysteine residues (Reng et al., 1993).

Amino acid sequence identities

The MDH amino acid sequences show divergence into 2 main phylogenetic groups of closely related enzymes (Fig. 1). Some of the most distantly related isozymes of MDH are found compartmentalized in different subcellular organelles of the same cell types. The sequence relationship of MDH isozymes from different sources is very complex. For example, MDHs from some eubacteria (*Escherichia coli*, *Salmonella typhimurium*) have relatively high sequence identity with the mitochondrial isozymes of eukaryotes, whereas other eubacterial MDHs (*Thermus* spp.) are more closely related to the cytoplasmic and chloroplast isozymes of eukaryotic MDH. The archaeal MDH from *Haloarcula marismortui* (Cendrin et al., 1993) has greater amino acid sequence identity with lactate dehydrogenase (LDH; EC 1.1.1.27), an enzyme with related structure and activity, than with other MDHs (Figs. 1, 2). This archaeal sequence suggests a link between the evolution of MDHs and LDHs. An explanation of the differences between MDHs from organelles and those from the cytosol is that a common ancestral *mdh* gene may have been duplicated before invasion of primordial eukaryotes by bacteria to produce mitochondria according to the probable endosymbiotic origin of these organelles (McAlister-Henn, 1988). The pattern of relatedness between MDHs is very complicated and a meaningful interpretation of the evolutionary origins of this enzyme still requires more amino acid sequence information.

Three-dimensional structure

The multiple amino acid sequence alignment of MDHs generally shows a low degree of primary structural similarity among the enzymes, in some cases as low as about 20% sequence identity (Fig. 2). Of the relatively few absolutely conserved residues a function has been assigned to several of these residues, which relate to nucleotide binding, catalysis, and the subunit interface (Birktoft & Banaszak, 1984). Some of the crucial residues are highlighted in Figure 2 and their importance is illustrated in Figure 3. Despite relatively low amino acid sequence identity, there is considerable structural identity between the crystal structures of MDH from *E. coli* (Hall et al., 1992; Hall & Banaszak, 1993), pig cytoplasm (Birktoft et al., 1989b), pig mitochondria (Roderick & Banaszak, 1986), and *Thermus flavus* (Kelly et al., 1993). These include the essential elements of secondary structure, location of the coenzyme binding site (Birktoft et al., 1989b), and the position of several catalytically important residues (Birktoft et al., 1982). Comparison of the crystal structure of *E. coli* MDH:citrate complex with pig cytoplasmic MDH:NAD^+ complex, for example, shows that the structures are essentially identical, although there is only about 20% sequence identity.

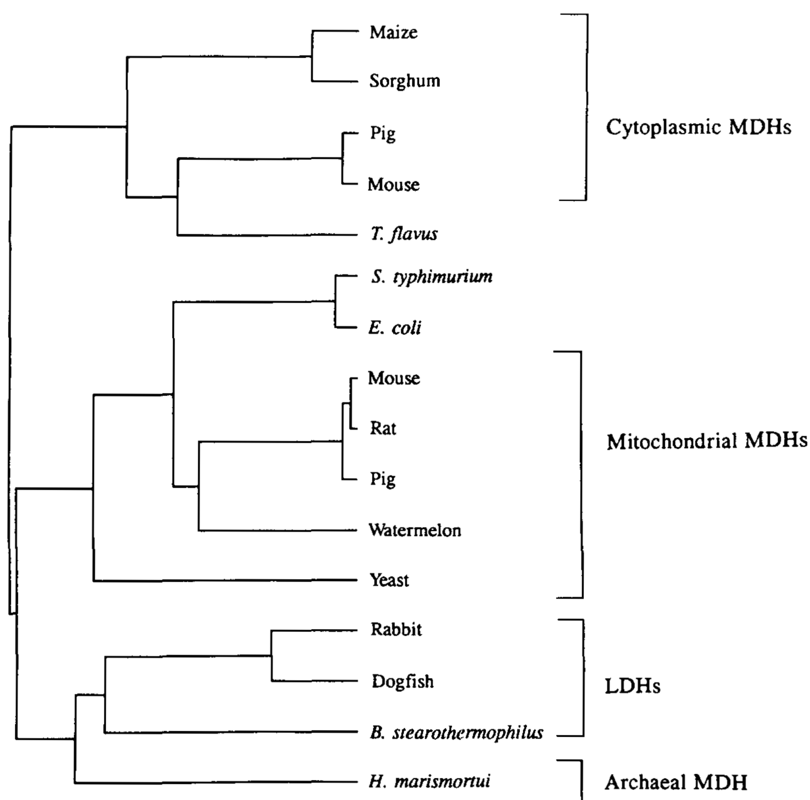

Fig. 1. Phylogenetic tree. A phylogenetic tree was constructed by using the program PILEUP. The following complete MDH amino acid sequences were available: maize (Metzler et al., 1989); sorghum (Luchetta et al., 1991); pig (c) (Birktoft et al., 1989b); mouse (c) (Setoyama et al., 1988); *T. flavus* (Nishiyama et al., 1986); *S. typhimurium* (Lu & Abdelal, 1993); *E. coli* (McAlister-Henn et al., 1987); mouse (m) (Takeshima et al., 1988); rat (m) (Grant et al., 1987); pig (m) (Birktoft & Banaszak, 1984); watermelon (Gietl et al., 1990); yeast (Minard & McAlister-Henn, 1991); *H. marismortui* (Cendrin et al., 1993). The LDH sequences used were rabbit chain-M (Sass et al., 1989) and dogfish chain-M (Eventoff et al., 1977) and *B. stearothermophilus* LDH (Barstow et al., 1986).

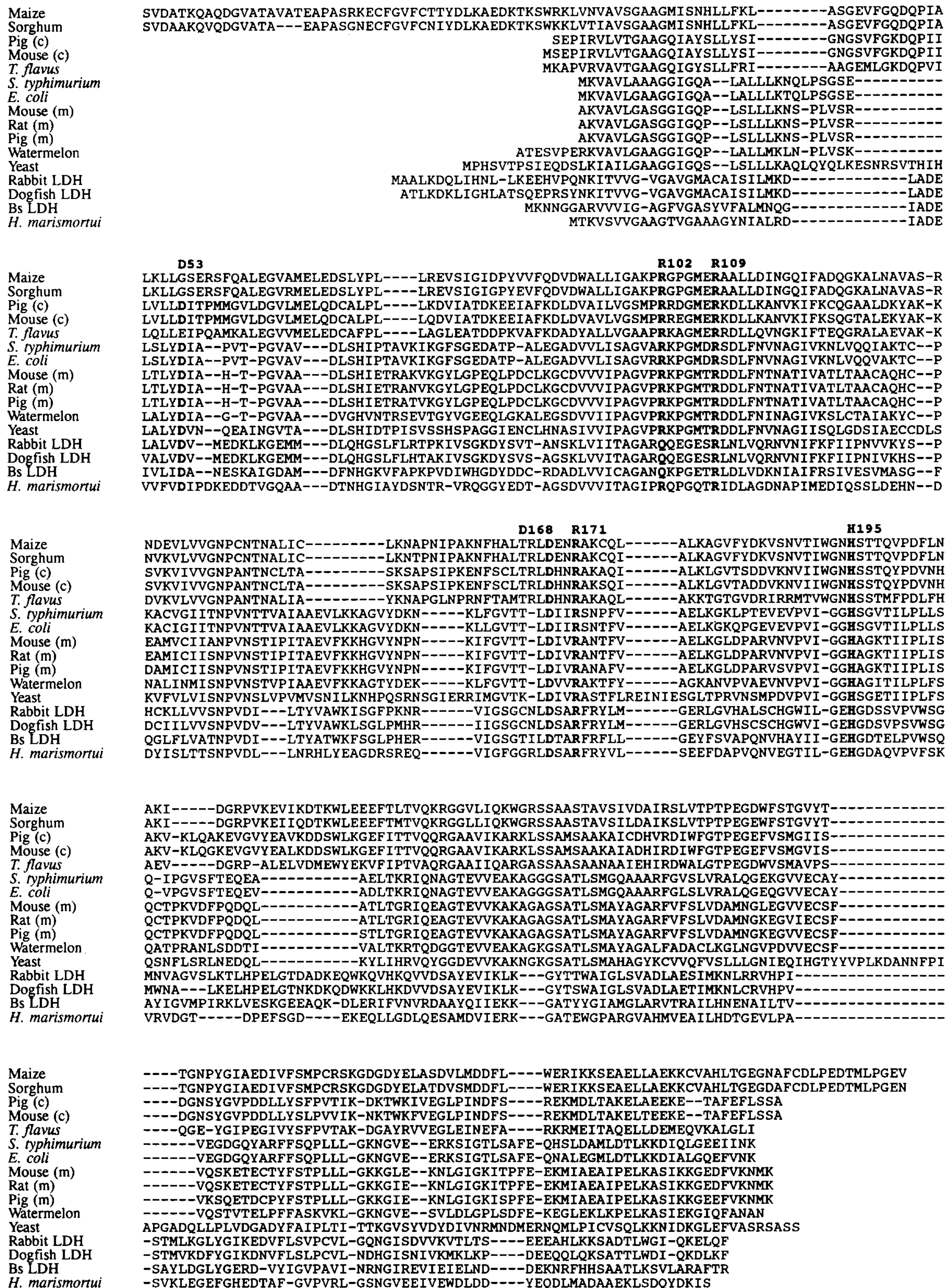

Fig. 2. Amino acid sequence alignments of malate dehydrogenases and lactate dehydrogenases. The MDH and LDH sequences were aligned by using the program PILEUP as implemented in the GCG package (Devereux et al., 1984). PILEUP creates a multiple sequence alignment from a group of related sequences by using a simplification of the method of pairwise alignments with the algorithm of Feng and Doolittle (1987). Where appropriate the MDH sequences are marked as mitochondrial (m) or cytoplasmic (c). The LDH sequences from rabbit, dogfish, and *B. stearothermophilus* (BsLDH) are shown for comparison. Residues important for catalysis and coenzyme binding are shown in bold and numbered according to the system for LDH (Eventoff et al., 1977). Gaps have been introduced to produce an optimal alignment.

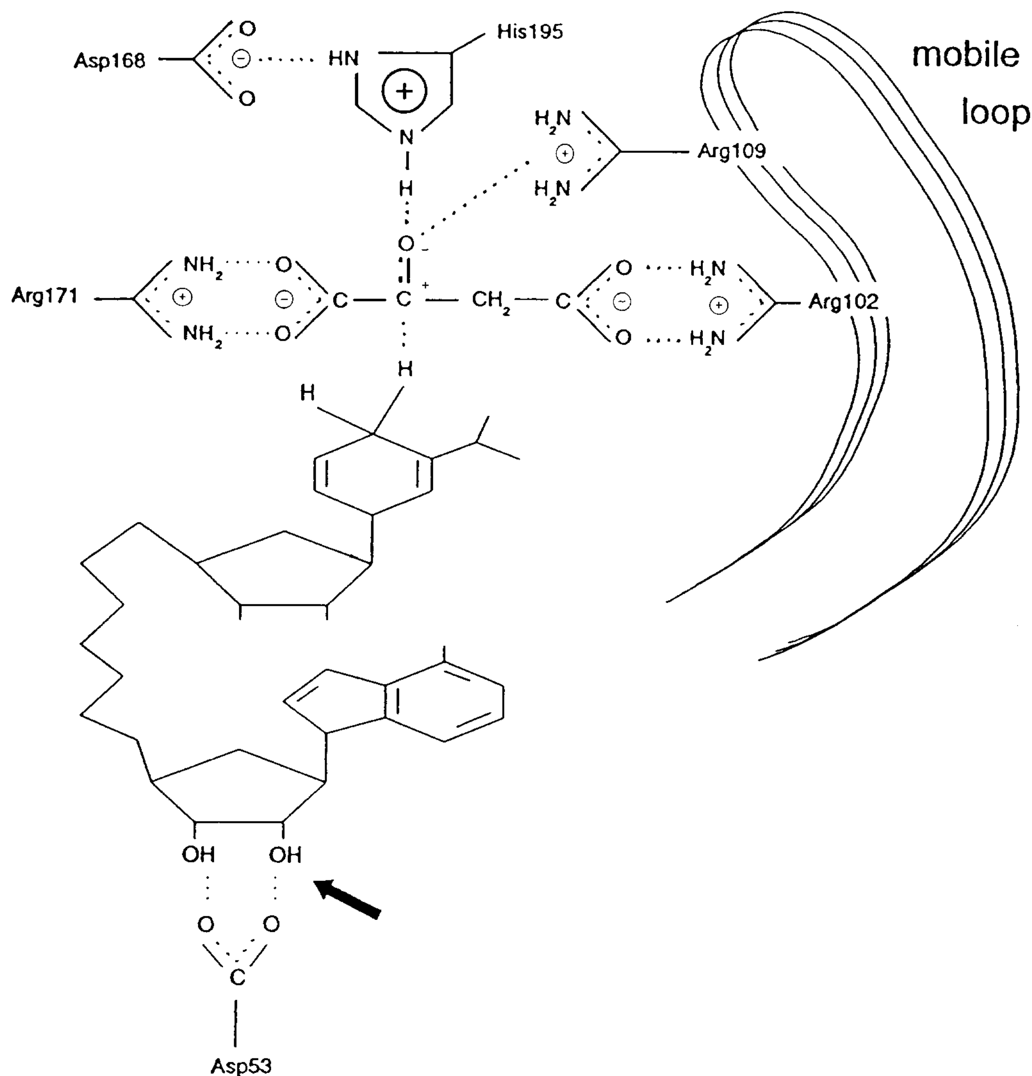

Fig. 3. Active-site vacuole of MDH. The schematic drawing summarizes the function of active-site residues with the substrate oxaloacetate and coenzyme NADH. The arrow indicates the position of phosphate in the case of $NADP^+$.

Catalysis

The active-site of MDH consists of a predominantly hydrophobic vacuole, which contains binding sites for the substrate and nicotinamide ring of the coenzyme. Upon formation of the enzyme:coenzyme:substrate ternary complex there is a protein conformational change in which an external loop closes over the active-site vacuole to screen the substrate and catalytically important residues from the solvent. In addition, other functionally important residues are brought into close proximity to the substrate (Clarke et al., 1986; Grau et al., 1981; Wigley et al., 1992). X-ray structures of MDHs and LDH crystallized in the presence and absence of substrate analogues, which bind to the active site, have identified 2 conformationally distinct forms of the enzyme with the external loop in either the up or down position. Real-time movement of this loop region, demonstrated in LDH by using fluorescence anisotropy measurements, was correlated with the rate-determining step in the reaction for *Bacillus stearothermophilus* LDH (Waldman et al., 1988). The loop region is highly conserved among MDHs (residues 98–110; Fig. 2), reflecting its crucial role in catalysis. There is additional evidence to support the existence of different molecular conformations of MDH that are involved at different stages of the catalytic cycle. The mobility of a fluorescent substrate analogue showed that binding of NAD^+ and NADH conferred 2 different conformational states on the substrate binding-site of mitochondrial MDH (Hönes, 1985). Similarly, using fluorescent coenzyme analogues, binding of substrate was found to induce a localized conformational change in the nicotinamide binding-site (Hönes et al., 1986).

One of the most striking similarities observed between the sequences and structures of MDH and LDH is the invariant and interacting H195 and D168 residues (Fig. 2). This histidine-aspartate pair form a proton relay system in the active site and allow the imidazole ring of the histidine to act as both an acid and a base (Birktoft & Banaszak, 1983). This facilitates catalysis, which strongly suggests that both MDH and LDH operate by a similar mechanism.

Modulation of coenzyme specificity

The side chain of residue 53 is important for coenzyme binding and specificity by hydrogen bonding with the adenosine ribose hydroxyl groups (Birktoft et al., 1989a, 1989b; Hall et al., 1992; Wigley et al., 1992; Kelly et al., 1993). Residue 53 is chemically conserved with an acidic side chain in all NAD^+-dependent MDHs, but is found as glycine in the $NADP^+$-dependent chloroplast enzymes of maize and sorghum (Fig. 2). Several workers have used sequence information from chloroplast MDH to engineer $NADP^+$ specificity into NAD^+-specific MDH and the related enzyme LDH. The specificity of *T. flavus* MDH was altered from NAD^+ to $NADP^+$ by substitution of 3 residues on a loop including residue 53 (Nishiyama et al., 1993). A natural $NADP^+$-specific enzyme has not yet been isolated for LDH, but this was artificially achieved for *B. stearothermophilus* LDH by the single amino acid substitution of D53S (Feeney et al., 1990). This shows that nucleotide binding characteristics can be altered in both LDH and MDH by a single amino acid change, and also shows that the same factors apply in both enzymes.

Modulation of substrate specificity

In contrast to most MDHs, eubacterial LDHs are subject to allosteric regulation by fructose 1,6-bisphosphate. The 3-dimensional X-ray structures for dogfish (Abad-Zapatero et al., 1987) and *B. stearothermophilus* (Wigley et al., 1992) LDH have led to an understanding of catalysis by these molecules.

There are 3 arginine residues (R102, R109, and R171) that are absolutely conserved in all MDHs and are important for substrate binding and catalysis (Figs. 2, 3). R102 and R109 are on the underside of the mobile loop and interact with substrate in the ternary complex. R109 has been shown to play a role in stabilization of the substrate transition state in LDH, which is thought to involve polarization of the substrate carbonyl bond, which is stabilized by R109 (Clarke et al., 1986). Conservation of this residue in MDH suggests that the mechanism of catalysis is very similar to that in LDH. The guanidinium side chains of R102 and R171 form counterions for the substrate carboxylate groups, which contribute to binding and orientation of the substrate in the active site (Fig. 3).

Rational redesign of MDH and LDH molecules has shown how substrate specificity of an enzyme can be redefined by a single amino acid change. The amino acid at position 102 is arginine in MDH and glutamine in LDH (Fig. 2). The preferred substrates are oxaloacetate for MDH and pyruvate for LDH (Fig. 4). An R102Q mutant enzyme of *E. coli* MDH had the effect of relaxing the high degree of specificity for the substrate oxaloacetate (Nicholls et al., 1992). These results were different from those for a Q102R mutant of *B. stearothermophilus* LDH where MDH specificity was conferred on the LDH enzyme framework (Wilks et al., 1988) and for an R102Q mutant of *H. marismortui* MDH where LDH activity was conferred on the MDH enzyme framework (Cendrin et al., 1993) (Fig. 5). The switching of specificity observed with the *B. stearothermophilus* LDH and *H. marismortui* MDH may reflect the close phylogenetic grouping of these enzymes (Fig. 1). The arginine residue of MDH appears to provide a mechanism that precludes catalysis of pyruvate. By introducing a charge imbalance in the ternary complex with an uncharged substrate this is of physiological importance to an organism because of the relatively high

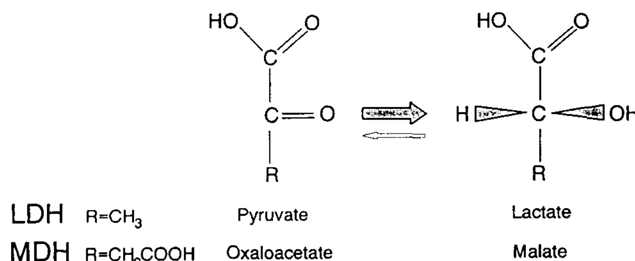

Fig. 4. Preferred substrates of MDH and LDH.

concentrations of pyruvate in vivo. R102 appears to be conserved in MDH as the result of a strong evolutionary pressure on an organism to develop a highly selective binding mechanism for oxaloacetate with a strong bias against oxaloacetate as the product.

The redesign of MDH by rational rather than random mutations is proving to be a useful method for study of catalysis of small substrates. One result of this work is redesign of *E. coli* MDH, which is highly specific for oxaloacetate, to an enzyme with broad specificity for 2-ketoacid substrates by a single mutation (R102Q) (Nicholls et al., 1992). The R102Q mutant enzyme may find industrial applications as a single reagent to perform 2-hydroxy acid chiral conversions. This has been made possible by knowledge of 3-dimensional structures of MDH and the related LDH. The relatedness of MDH and LDH has been shown by sequence alignments, phylogenetic relationships, and 3-dimensional structures. Sequence identity between MDHs and LDHs has allowed information from one enzyme to be extrapolated to other enzymes and facilitates the prediction of important residues. With the information gained on MDH the result is an enzyme framework that may be modified to suit a particular 2-hydroxyacid substrate for use as an industrial catalyst or as a diagnostic reagent and this may be obtained by rational re-

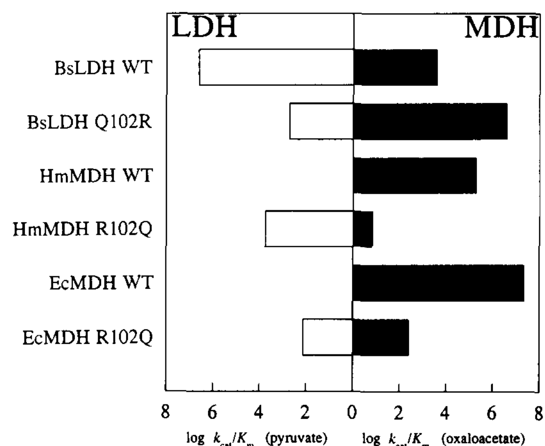

Fig. 5. Influence of the residue at position 102 on substrate selection. The data show efficiency, expressed as log k_{cat}/K_m, of wild-type and mutant enzymes for the natural substrate of MDH (oxaloacetate) and LDH (pyruvate). The enzymes used were from *B. stearothermophilus* LDH (BsLDH) (Wilks et al., 1988), *H. marismortui* MDH (HmMDH) (Cendrin et al., 1993), and *E. coli* (EcMDH) (Nicholls et al., 1992).

design of the enzyme catalytic properties. This process is simplified by the knowledge that change of very few or single amino acid residues in MDH and LDH may result in profound changes in specificity for either substrate or coenzyme.

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