

## Minireview

## Malate Dehydrogenase: Distribution, Function and Properties

R. A. MUSRATI, M. KOLLÁROVÁ, N. MERNIK AND D. MIKULÁŠOVÁ

*Department of Biochemistry, Faculty of Natural Sciences, Comenius University,  
Mlynská dolina CH-1, 842 15 Bratislava, Slovak Republic*

**Abstract.** Malate dehydrogenase (MDH) (EC 1.1.1.37) catalyzes the conversion of oxaloacetate and malate. This reaction is important in cellular metabolism, and it is coupled with easily detectable cofactor oxidation/reduction. It is a rather ubiquitous enzyme, for which several isoforms have been identified, differing in their subcellular localization and their specificity for the cofactor NAD or NADP. The nucleotide binding characteristics can be altered by a single amino acid change. Multiple amino acid sequence alignments of MDH show that there is a low degree of primary structural similarity, apart from several positions crucial for catalysis, cofactor binding and the subunit interface. Despite the low amino acids sequence identity their 3-dimensional structures are very similar. MDH is a group of multimeric enzymes consisting of identical subunits usually organized as either dimer or tetramers with subunit molecular weights of 30–35 kDa. MDH has been isolated from different sources including archaea, eubacteria, fungi, plant and mammals.

**Key words:** Malate dehydrogenase — Isoenzymes — Protein structure

Malate dehydrogenase is a widely distributed enzyme. The preferred substrate for MDH is oxaloacetate (Nicholls et al. 1992). It plays a crucial role in many important metabolic pathway including the tricarboxylic acid cycle, glyoxylate bypass, amino acid synthesis, gluconeogenesis and facilitation of exchange of metabolites between cytoplasm and subcellular organelles.

MDH is a multimeric enzyme consisting of identical subunits usually organized as either dimers or tetramers with subunit molecular weights of 30–35 kDa. MDH from *Nitzschia alba* is composed of eight identical subunits. It is the only octameric MDH so far reported (Yueh et al. 1989). Each subunit of MDH contains a dinucleotide-binding domain which is similar in sequence and structure to that of

Correspondence to: Marta Kollárová, Department of Biochemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia  
E-mail: [kollarm@fns.uniba.sk](mailto:kollarm@fns.uniba.sk)

other nicotinamide-nucleotide dependent enzymes. X-ray structures of MDH show the bound cofactor to be in an extended conformation (Hill et al. 1972; Birktoft et al. 1989b), and similar to the conformation of cofactor bound to lactate dehydrogenase (LDH) (Piontek et al. 1990).

Malate dehydrogenase isoenzymes can serve as a model system for studying the evolution, protein sorting to different cell compartments, and for comparison of MDH isoenzymes.

Eukaryotic tissue contains multiple forms of MDH involved in different metabolic pathways and located in different subcellular compartments. MDH in eukaryotic organisms is found in microbodies such as glyoxysomes and peroxisomes, in the mitochondria, in the cytoplasm and in chloroplasts. All MDHs are NAD-dependent except the chloroplastic enzyme which requires NADP as a cofactor.

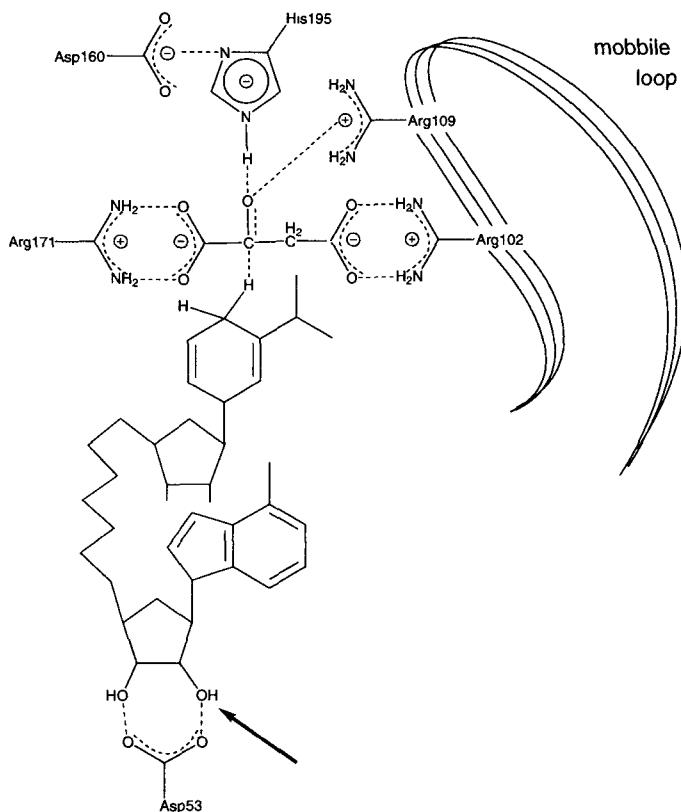
*Escherichia coli* has only one form of MDH (Hall et al. 1992). Its MDH is most similar to eukaryotic mitochondrial MDH which has 59% sequence identity and a similar tertiary structure. Two isoenzymes of MDH, mitochondrial and cytoplasmic, are present in mammalian cells. In higher plants, there are three NAD-dependent forms located in the cytoplasm, the mitochondria and the microbodies, and one NADP-dependent form present in chloroplasts (Poeydomenge et al. 1995). The different isoenzymes are encoded in genes of the nucleus, and they are synthesized on cytoplasmic ribosomes and imported to their respective organelles.

In principle, different isoenzymes can be derived from unrelated genes, from genes in the same family sharing a common ancestral gene or by alternative RNA splicing and posttranslational modification from a single gene.

The MDH amino acid sequences show divergence into two main phylogenetic groups of closely related enzymes – cytoplasmic and mitochondrial MDHs. Some of the most distantly related isoenzymes of MDH are found compartmentalized in different subcellular organelles of the same cell types (Goward and Nicholls 1994). The archaeabacterial sequence of *Haloarcula morismortui* (Cendrin et al. 1993) suggests a link between the evolution of MDHs and lactate dehydrogenases (LDHs). An explanation of the difference 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 et al. 1987).

## Catalysis

The active site of MDH consists of a predominantly "hydrophobic vacuole", which contains binding sites for the substrate and the 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. In addition, other functionally important residues are brought into close



**Figure 1.** 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<sup>+</sup> (Goward and Nicholls 1994).

proximity of the substrate (Grau et al. 1981; Clarke et al. 1986; Wigley et al. 1992). The loop is highly conserved among MDHs (residues 98–110), reflecting its crucial role in catalysis (Fig. 1). X-ray structures of MDHs 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 external loop in either the up or down position. The active site of this enzyme contains invariant and interacting H195 and D168 residues. His-Asp pair linked by a hydrogen bond may function as a proton relay system during catalysis and allow the imidazole ring of the histidine to act as both an acid and a base. This pair could also provide an explanation for the relatively stronger binding by cytoplasmic MDH of NADH versus NAD (Birktoft and Banaszak 1983).

The side chain of residue D53 is important for coenzyme binding and specificity by hydrogen bonding with the adenosine ribose hydroxyl groups. It is chemically conserved with an acidic side chain in all NAD-MDHs. G53 was found in the chloroplastic NADP-MDH of maize and sorghum (Birktoft et al. 1989a; Hall et al. 1992; Wigley et al. 1992; Kelly et al. 1993). There are 3 arginine residues (R102, R109 and R171) which are important for substrate binding and catalysis. R102 and R109 are on the underside of the mobile loop and interact with substrate in ternary complex. The quanidium 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 (Clarke et al. 1986; Goward and Nicholls 1994).

### Mitochondrial MDH

The majority of mitochondrial proteins are encoded in the nucleus, synthesized in the cytoplasm and imported to one of four locations in the mitochondria: the matrix, the intermembrane space, the outer membrane or the inner membrane. Many mitochondrial proteins are synthesized as precursors, which are imported post-translationally with the aid of nucleotide triphosphate hydrolysis and the electrochemical potential across the inner membrane. Many precursors of matrix proteins such as malate dehydrogenase contain N-terminal presequences (transit sequences) of 10–70 residues in length, which are cleaved off by specific peptidases after import to the mitochondria. Import is dependent on the precursor protein being in an unfolded state, and it requires membrane potential across the inner membrane (Hartl et al. 1989). There is evidence that import is mediated by proteins in the cytosol, receptors in the membrane and chaperons in the matrix (Schatz 1993). Transit sequences are relatively rich in positively charged residues (mainly arginine), lack acidic side-chain and have a high content of serine and threonine and small clusters of adjacent hydrophobic groups (MacLachlan et al. 1994).

Comparison of watermelon mMDH with mammalian, yeast and *E. coli* MDH gives 55–60% overall identity of residues (McAlister-Henn et al. 1987). On the other hand, the amino acid sequences of mouse mMDH and cytosolic MDH (cMDH) show only 23% overall identity (Joh et al. 1987). Surprisingly, comparisons of the amino acid sequences among the eukaryotic and bacterial MDHs revealed that the similarity between the mMDHs from plants, yeast, *E. coli* and the thermophilic bacterium *Thermus flavus* exceeds the intraspecies sequence similarity between mitochondrial and cytoplasmic MDH (Nishiyama et al. 1986).

The structure of mitochondrial MDH is well defined. In most organisms, it is a dimer composed of identical subunits with approximate molecular weights of 34 kDa (Banaszak and Bradshaw 1975), and the known amino acid sequence contains 314 residues. The gene encoding the mitochondrial isoenzyme of MDH is present in a single copy in genomic DNA of *S. cerevisiae*. The specific activity of

mMDH was elevated approximately eight-times in yeast cells transformed with a multicopy plasmid containing the entire encoding region for the corresponding gene (McAlister-Henn et al. 1987). Although several collections of yeast mutants with defects in mitochondrial functions and oxidative energy metabolism have been isolated, mutants with specific defects in mitochondrial MDH have not been described (Parker and Mattoon 1969; Tzagoloff et al. 1975; Ciriacy 1977).

Mitochondrial MDH is involved in three different pathways: 1. tricarboxylic acid cycle, 2. conversion of glycine to serine to provide reducing equivalents, and 3. supply of CO<sub>2</sub> for fixation in bundle sheath chloroplasts of higher plants.

In the tricarboxylic acid cycle mitochondrial MDH provides, together with isocitrate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase, for NADH to be oxidised in the respiratory chain, while succinate dehydrogenase yields FADH<sub>2</sub>. Plant mitochondria preferentially oxidise NADH produced in the matrix space as a result of substrate oxidation. The latter two pathways suggests cooperation of the mitochondrion with the peroxisome in photorespiration and with the chloroplast in the concentration mechanism of CO<sub>2</sub> for C<sub>4</sub> photosynthesis, respectively (Gietl 1992).

In mammalian and yeast cells mitochondrial and cytosolic MDHs there are components of the malate/aspartate shuttle; they represent an important mechanism for exchange of substrates and reducing equivalents between metabolic pathways separated by the mitochondrial membrane. NADH is formed in the cytoplasm during glycolysis and is required for respiration and a variety of metabolic processes. From the cytoplasm to the mitochondria it is transported across the mitochondrial membrane by the malate/aspartate shuttle because intact mitochondria are impermeable for NADH (Setoyama et al. 1990).

### Cytosolic MDH

When the mitochondrial isoenzyme is synthesized as higher molecular weight precursor, the *in vitro* translation of the cytosolic MDH yields a product which has the same molecular weight as the native isoenzyme.

Eukaryotic cells contain two forms of MDH enzymes – one in the mitochondria and the other one in the cytoplasm. Both forms of MDH are synthesized in the cytoplasm, where cMDH remains after acetylation of the N-terminal residue. mMDH is synthesized with an N-terminal extension residue and is subsequently imported into the mitochondrial matrix (Chien and Freeman 1984). Cytosolic MDH is a homodimer, each subunit having a molecular mass of 35 kDa and containing 332 amino acids of known sequence (Birktoft et al. 1987; Joh et al. 1987).

One of the more noticeable differences between cMDH and mMDH is the difference in the overall polarity of the two enzymes. cMDH is more polar as well as more acidic than mMDH, and this difference is due solely to the difference in the

number of charged residues. There are 41 basic residues (31 lysines + 10 arginines) and 43 acidic residues (25 aspartates + 18 glutamates) in cMDH. In mMDH there are 25 lysines and 8 arginines for total of 33 basic residues, and 13 aspartates and 16 glutamates for a total of 29 acidic groups (Birktoft et al. 1989a).

The sequence identity between cMDH and mMDH is relatively low, being of the order of about 20–25%. The best fit of the molecular structure of cMDH to that of lactate dehydrogenase has been obtained by the least square method. This similarity between the dimeric cMDH and the tetrameric LDH reported earlier by Rao and Rossmann (1973), particularly in the nucleotide binding domain, has been confirmed. The active sites of these two enzymes contain similarly oriented His-Asp pairs linked by a hydrogen bond which may function as a proton relay system during catalysis. This pair could also provide an explanation for the relatively stronger binding by cMDH and LDH of NADH versus NAD.

### Peroxisomal and glyoxysomal MDH

Peroxisomes are organelles present in almost all eukaryotic cells. Like the mitochondrion, the peroxisome is a major site of oxygen utilization. In fact, the peroxisome is thought by some to represent the vestige of an ancient organelle that carried out all of the oxygen metabolism of primitive preeukaryotic cells when oxygen entered the atmosphere. Most peroxisomes catalyze the breakdown of fatty acids to acetyl-CoA using a special H<sub>2</sub>O<sub>2</sub>-producing enzyme. The acetyl-CoA produced can be transported via the cytosol to the mitochondria to feed the citric acid cycle, or it can be used for biosynthetic reactions elsewhere.

Two very different types of peroxisomes have been extensively studied in plants. One type is present in leaves, where it catalyzes the oxidation of a side product of the reaction that fixes CO<sub>2</sub> in carbohydrate (photorespiration). A very different type of peroxisome is present in germinating seeds, where it serves to convert the fatty acids stored in seed lipids into sugars needed for the production of the materials of the young plant. Because this is accomplished by a series of reactions known as the glyoxylate cycle, these peroxisomes are also called glyoxysomes.

Metabolism of glycolate carbon occurs sequentially in three organelles, the peroxisomes, the mitochondria and the chloroplasts (Lorimer and Andrews 1980). In the peroxisome glycolate is oxidised to glyoxylate and then transaminated to glycine with either glutamate or serine (Rehfeld and Tolbert 1972). Glycine then stays in the peroxisome and is oxidised to ammonia, CO<sub>2</sub> and serine in the mitochondrion. Serine is converted to glycerate by serine:glyoxylate aminotransferase and hydroxypyruvate reductase in the peroxisome, and in this form photorespiratory carbon returns to the chloroplast. Glycerate is phosphorylated to phosphoglycerate by glycerate kinase, and can reenter the photosynthetic cycle.

Photorespiration is an example for the tight cooperation of metabolic pathways

located in different cell compartments. A malate/oxaloacetate/aspartate shuttle has been proposed for plant microbodies similar to that for mitochondria (Dry et al. 1987). Leaf peroxisomes as well as seed glyoxysomes contain a large amount of activity of an isoenzyme of NAD-malate dehydrogenase (Yamazaki and Tolbert 1969; Curry and Ting 1973). Besides catalase (an enzyme that constitutes up to 40 percent of the total peroxisomal protein), malate dehydrogenase is the most active enzyme in plant microbodies. It is therefore not surprising that enzymes present in both glyoxysomes and peroxisomes are identical. Peroxisomal and glyoxysomal MDHs are serologically indistinguishable and have the same isoelectric points and function as dimers (Hock and Gietl 1982).

The transport of glyoxysomal MDH from the site of synthesis in the cytoplasm to the site of function in the organelle involves translocation of the protein across the single membrane of the organelle. Glyoxysomal MDH is very similar to mitochondrial and cytoplasmic MDHs and also lactate dehydrogenases. Especially the amino acids Arg-87, Gly-185 and Gly-228 which are crucial for selecting malate as substrate, are rigorously conserved in MDH enzymes, while in LDH enzymes an equally strong conservation is observed for Gln, Asp and Thr in the same positions (Wilks et al. 1988).

Glyoxysomes lack DNA, they are surrounded by a single membrane and share at least two biochemical capabilities with peroxisomes of animals and fungi: O<sub>2</sub> processing (based on the conversion of H<sub>2</sub>O<sub>2</sub> by catalase) and fatty acid oxidation. In glyoxysomes they convert the long-chain fatty acids to their CoA esters (Cooper 1971). Their membranes contain an alkaline lipase that hydrolyses triacylglycerols (Muto and Beevers 1974; Huang 1987). They possess a complete set of enzymes for the  $\beta$ -oxidation of fatty acids, and enzymes of the glyoxylate cycle. The  $\beta$ -oxidation of fatty acids ends up with the production of acetyl-CoA. The enzymes of the glyoxylate cycle catalyze the conversion of two molecules of acetyl-CoA to succinate, and comprise citrate synthase, aconitase, isocitrate lyase, malate synthase and malate dehydrogenase enzymes (Beevers 1969). For the fate of NADH synthesized within the glyoxysomes which do not contain an electron transport system linking NADH to O<sub>2</sub>, two possibilities are discussed: 1. oxidation of NADH in glyoxysomes by a malate-aspartate shuttle (Mettler and Beevers 1980) would involve transport of malate from the glyoxysomes to mitochondria, and 2. the alternative possibility is coupling of  $\beta$ -oxidation of fatty acids and glyoxylate cycles to NADH:cytochrome c and ferricyanide reductases in glyoxysomes which may allow  $\beta$ -oxidation and the glyoxylate cycle to be partially uncoupled from mitochondrial oxidative phosphorylation (Donaldson and Fang 1987).

### **Chloroplastic MDH**

Besides the NAD-dependent isoforms of malate dehydrogenase located in microbod-

ies, the mitochondria and the cytoplasm, an NADP-dependent form of the enzyme is found in the chloroplasts in higher plants. NADP-dependent MDH (NADP-MDH) (EC.1.1.1.82) is involved in the C<sub>4</sub> dicarboxylic acid cycle responsible for the primary fixation and transfer of CO<sub>2</sub> in C<sub>4</sub> plants. It is also present in C<sub>3</sub> type plants where it is implicated in chloroplast shuttle mechanisms which might help export reducing power.

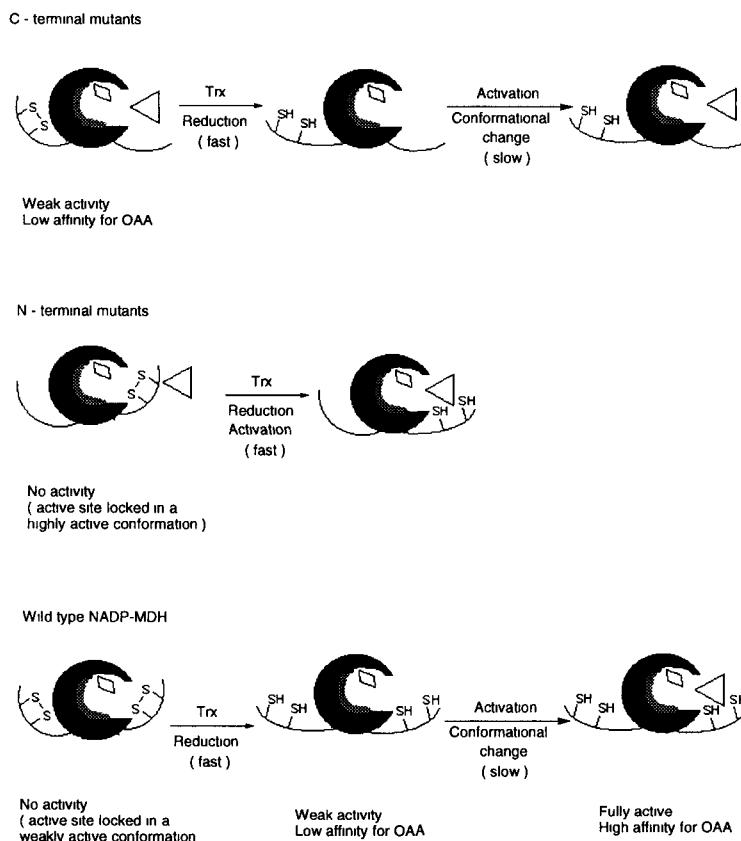
NADPH-MDH is a model enzyme in that its activity is strictly controlled by light (Johnson and Hatch 1970). Indeed, NADP-MDH is inactive in the dark and gets activated by light via the ferredoxin – thioredoxin system (Wolosiuk et al. 1980).

NADP-MDH resembles the non-regulatory NAD-MDH, except for two sequence additions, one N-terminal and one C-terminal. Due to the presence of both N- and C-terminal extensions chloroplastic MDHs exhibit larger molecular masses, and they represent crucial parts of the protein for redox regulation.

Several cysteine residues are located in the NADP-MDH polypeptide, and all are specific to this chloroplastic redox regulatory isoform. Two of these are located in the N-terminal extension sequence and one in the C-terminal extension. Chemical derivatization followed by sequence analysis in sorghum (Decottignies et al. 1988) has shown that there is a light-dependent reducible disulphide bridge present in the N-terminal extension. Site-directed mutagenesis has indeed confirmed that there is a thioredoxin-reducible disulphide bridge involving these two residues (Issakidis et al. 1992), but has also shown that there is a second disulphide needed for regulation. Additional site-directed mutagenesis experiments have succeeded in creating a redox insensitive NADP-MDH, by mutation of the N-terminal disulphide, together with either or both of the most C-terminal cysteines (C377 and/or C365 in sorghum), indicating that these cysteines could constitute the second regulatory disulphide (Issakidis et al. 1993; 1994). There is experimental evidence that the disulfide bridge involving the C-terminal residues is shielding access to the catalytic residues, and that the N-terminal end is involved in the slow conformational change of the active site needed for activation (Issakidis et al. 1996).

On the basis of the results of mutagenesis experiments and three-dimensional structure modeling of the chloroplastic isoenzyme, Issakidis et al. (1994) proposed a model for the mechanism of activation of NADP-MDH (Fig. 2).

Chloroplast NADP-MDH possesses a His-Asp pair at the active site which probably forms a proton relay system. The involvement of such a His/Asp pair in catalysis has already been described for LDH and NAD-MDH. It is acting as an acid in the reduction of the keto-acid and as a base in the oxidation of the hydroxy-acid (Birktoft and Banaszak 1983). So, His-229 and Asp-201 play a crucial role in the catalytic mechanism of chloroplastic NADP-MDHs (Lemaire et al. 1996). Sequence data of NADP-MDH from pea (Reng et al. 1993), sorghum (Cretin et al. 1990), maize (Metzler et al. 1989) and ice-plant are available.



**Figure 2.** Schematic model of activation mechanism of NADP-MDH (either wild type or mutant). Only one subunit is shown. The coenzyme binding site is symbolized by the diamond, as it is accessible from the surface. The triangles represent oxaloacetate. The N-terminal extention is to the left of the subunit and the C-terminal is to the right, close to the active site. *Trx*, thioredoxin (Issakidis et al. 1994).

NADP-MDH is an essential component of the malate/oxaloacetate shuttle, which balances reducing equivalents between the chloroplast and the cytosol. In C<sub>4</sub> plants, NADP-MDH activity is 10-times higher and acts to convert oxaloacetate to malate in chloroplasts of mesophyll cells for transport to bundle sheath cells (Hatch and Slack 1969). During C<sub>4</sub> photosynthesis, atmospheric CO<sub>2</sub> is fixed by carboxylation of phosphoenolpyruvate in mesophyll cells (by PEP carboxylase), giving C<sub>4</sub> dicarboxylic acids which are decarboxylated in bundle sheath cells by one of three different decarboxylases: NADP-malic enzyme, NAD-malic enzyme or PEP carboxykinase. When malate is transported to bundle sheath cells and decar-

boxylated via NADP-malic enzyme, it acts as a carrier of reducing power as well as CO<sub>2</sub>. The NADPH formed is directly utilised for phosphoglycerate reduction.

The encoded amino acid sequence of maize predicts that NADP-MDH is synthesized as a preprotein of 432 amino acids and processed into a mature protein of 375 amino acids with removal of a 57 amino acids long transit peptide. The sorghum enzyme is synthesized as a precursor of 429 amino acids and gets imported into the chloroplast where it is processed to a mature subunit of 389 amino acids. Despite the lack of sequence similarities to other chloroplast transit peptides the extra sequence shows the common features : it is rich in the hydroxylated amino acids serine and threonine (14%), it is also rich in small hydrophobic amino acids such as alanine and valine (28%), it shows net positive charge (8 arginines, 1 lysine), and is generally deficient in acidic amino acids (2 aspartates). The maize enzyme is similar to other MDHs in regions related to enzymatic function (Wilks et al. 1988).

The similarity of the C<sub>3</sub> and C<sub>4</sub> forms of NADP-MDH suggests that genes for C<sub>4</sub> enzymes may have been recruited from existing genes encoding C<sub>3</sub> enzymes (Gietl 1992).

### Eubacterial MDH

*Escherichia coli* malate dehydrogenase (eMDH) is a homodimer and it is comprised of 312 amino acid residues per subunit (Sutherland and McAlister-Henn 1985; McAlister-Henn et al. 1987). The structures of cytosolic and mitochondrial MDHs from porcine heart have been determined (Roderick and Banaszak 1986; Birktoft et al. 1989a) and a comparative study between these enzymes and eMDH demonstrated that eMDH has 58% sequence identity with mMDH, but only about 20% identity with cMDH (Hall et al. 1992; Gleason et al. 1994).

In the crystalline structures of 2 prokaryotic and 2 eukaryotic forms, the sub-unit interfaces are conformationally homologous. To determine whether or not the quaternary structure of MDH is linked to catalytic activity, mutant forms of the enzyme from *E. coli* have been constructed. Utilizing the high-resolution structure of *E. coli* MDH, the dimer interface was analyzed critically for side chains that were spatially constricted and needed for electrostatic interactions. Two such residues were found, D45 and S226. At the nearest point in the homodimer, they were found in different subunits, hydrogen bonded across the interface, and did not interact with any catalytic residues. Each residue was mutated to a tyrosine, which should disrupt the interface because of its large size. All mutants were cloned and purified to homogeneity. Gel filtration of the mutants showed that D45Y and D45Y/S226Y were both monomers, whereas the S226Y mutant remained a dimer. The monomeric D45Y and D45/S226Y mutants had 14,000 and 17,500-times less activity, respectively, than the native enzyme. The dimeric S226Y had only 1.4-times less specific activity.

Crystallographic studies have shown that the dimer interface consists mainly of interacting  $\alpha$ -helices that fit compactly together. The active sites in these dimeric proteins are well separated from each other; they are not in the subunit interface and the bound dicarboxylic acid substrates are about 3 nm apart. The 3-dimensional subunit-subunit relationship is the same in all the known MDH structures and in one of the interfaces present in the tetrameric lactate dehydrogenases.

**Table 1.** Homology within a portion of the  $\alpha$ -helical region in the dimer interface In *E. coli* MDH, D45 and H48 have the most extensive hydrogen bonding pattern Residue numbers correspond to *E. coli* MDH (Hall et al 1992)

<i>E. coli</i> MDH	G	V	A	V	D	L	S	H	T	L	S	M	G
Yeast mMDH	G	V	A	T	D	L	S	H	T	L	S	M	A
Pig/mouse mMDH	G	V	A	A	D	L	S	H	T	L	S	M	A
Pig cMDH	G	V	L	M	E	L	Q	D	A	M	S	A	A
tMDH	G	V	V	M	E	L	R	D	A	A	S	A	A
PigLDHA, LDHB	G	E	M	M	D	L	Q	H	G	L	S	V	A
	41					45	48	224			226		228

Some researchers have suggested the dimer structure to be critical for enzymatic activity. Several highly conserved amino acid residues are found in this interface (Table 1), with D45 and H48 having the most extensive hydrogen bonding interactions (Breiter et al. 1994). The stability of the subunit-subunit interface in eMDH is the result of direct hydrogen bonds, water-mediated hydrogen bonds and hydrophobic contacts.

Two schools of thought arose surrounding the structure-function relationship (Harada and Wolfe 1968). The first proposed the reciprocating compulsory order mechanism where each subunit alternates as the "active" and the "helper" subunit, but both are needed for activity. This mechanism predicts an inactive monomer, and was confirmed by studies that showed a dramatic reduction of enzyme activity on dissociation to monomers at low enzyme concentration, at pH 5.0 and in the absence of substrate (Bleile et al. 1977; Wood et al. 1981a,b). The second mechanism introduces an equilibrium between two conformers of MDH, one of which preferentially binds citrate and NAD, whereas the other binds NADH. This would suggest an active monomer (Mullinax et al. 1982).

From termophilic organism *Thermus flavus* MDH (tMDH) has been purified and its enzymatic properties have been analyzed (Iijima et al. 1980). It is a dimer enzyme composed of two identical subunits, each of a molecular weight of 35 kDa (Iijima and Saiki 1984). Nishiyama et al. (1986) have cloned the gene for the *T.*

*flavus* malate dehydrogenase (tMDH) into *E. coli* and reported the sequence. Interestingly, the prokaryotic tMDH sequence is more identical to the eukaryotic cMDH (55%) than to mMdh (20%). Random mutagenesis of *T. flavus* yielded a mutant strain that possessed 3-times higher MDH activity than the wild type strain. The MDH gene cloned from the mutant strain was found to encode an altered enzyme with a single amino acid exchange of Ile for Thr-190 which caused an increase in the apparent enzyme activity and loss of substrate inhibition in the presence of an excess of oxaloacetate (Nishiyama et al. 1991).

The position of the NAD-binding loop relative to the body of the protein in tMDH was compared to that in cMDH and LDH; it was observed that the tMDH-NAD binary complex is more similar to that in LDH than to that in the cMDH-NAD binary complex (Kelly et al. 1993).

The malate dehydrogenase from grampositive bacteria *Streptomyces aureofaciens* has been purified and the molecular and catalytic properties of the enzyme have been studied. The protein is a homodimer, with a 38 kDa subunit molecular mass. The enzyme is very similar in many respects to other bacterial MDHs with the notable exception of a lack of inhibition by excess substrate. The enzyme is much more efficient in reducing oxaloacetate than in oxidating of malate (Mernik et al. 1998; Mikulášová et al. 1998).

### Archaeabacterial MDH

The limits of viability of organisms in their natural habitat are determined by extremes of temperature ( $-5^{\circ}\text{C}$  to  $110^{\circ}\text{C}$ ), hydrostatic pressure ( $\leq 120 \text{ MPa}$ ), pH (0–12) and water activity ( $\leq 5.5 \text{ mol/l NaCl and KCl}$ ). Adaptation to these extremes during the evolution led to development of thermophiles, barophiles, acidophiles, halophiles, etc. Numerous investigations of the molecular mechanism of adaptation at the genomic, metabolic and cellular level have not indicated a general strategy (Jaenicke 1981, 1988; Hecht et al. 1989). Comparison of homologous enzymes from species adapted to widely different conditions have revealed that ligand affinities seem to be conserved. The kinetic analog of this conservation of “corresponding states” would be compensatory effects via adjustments of activation free energies. To give an example, it has been established that cold and warm-adapted organisms show similar metabolic rates when the comparison is based on the temperature of their natural habitats (Hochachka and Somero 1973; Somero and Low 1976; Jaenicke 1981).

Halophilic eukaryotes and eubacteria overcome the extracellular osmotic pressure by accumulating “osmoprotectants” (Yancey et al. 1982). On the other hand, halophilic archaeabacteria accumulate salt (particularly  $\text{K}^+$  ions) at concentrations that can reach and exceed saturation (Christian and Walther 1962). The biochemical machinery of these prokaryotes has, therefore, been adapted in the course of

evolution to be able to function at salt concentrations at which most biochemical systems will cease to function. The biochemical and biophysical properties of several halophilic enzymes were studied in great detail (Eisenberg et al 1992).

The enzyme malate dehydrogenase of the extremely halophilic archaebacteria *Haloarcula marismortui* (hMDH) has been studied most extensively using a wide range of biochemical and biophysical methods (Pundak and Eisenberg 1981, Pundak et al 1981, Zaccai et al 1989). It was found that the enzyme is only stable in highly concentrated solutions of certain salts. The gene encoding hMDH could be isolated and sequenced. The enzyme is composed of 303 amino acids and its molecular mass is 32,638 Da. The deduced amino acid sequence of the enzyme was found to be more similar to the sequence of L-lactate dehydrogenase (LDH) from various sources than to the sequence of other MDHs. The structural gene was cloned in the *E. coli* expression vector pET11a, and large amounts of a soluble but inactive form of the enzyme were produced upon its induction. Activation of the enzyme was obtained by increasing the salt concentration to 3 mol/l NaCl (Cendrin et al 1993). Both the halophilic and non-halophilic (mitochondrial) MDH show a close similarity in their  $K_m$  profiles. On other hand, the catalytic efficiency of the two homologous enzymes varies dramatically; halophilic malate dehydrogenase exhibits deactivation at low salt (Hecht et al 1989).

42 residues of the N-terminal amino acid sequence of MDH from thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* have been determined. In the archaebacterial enzyme the residue Gly(7), Gly(11) and Asp(33) are also present. The data suggest that in the enzyme from *S. acidocaldarius* like in the other MDHs the binding domain for NAD(H) is localized at the N-terminal part of the polypeptide chain (Gorisch and Jany 1989).

MDH from the extremely thermophilic methanogen *Methanothermus fervidus* has been isolated and its properties were characterized. This enzyme is a homomeric dimer with a molecular mass of 70 kDa displaying low specificity for NAD or NADP (Honka et al 1990).

## References

- Banaszak L J, Bradshaw R A (1975) Malate dehydrogenase. In: The Enzymes, 3rd ed (Ed P Boyer), 11, pp 369–396, Academic Press, New York.
- Beevers H (1969) Glyoxysomes of castor bean endosperm and their relation to glycogenesis. Ann NY Acad Sci 168, 313–324.
- Birktoft J J, Banaszak L J (1983) The presence of a histidine-aspartic acid pair in the active site of 2-hydroxyacid dehydrogenases. J Biol Chem 258, 472–482.
- Birktoft J J, Bradshaw R A, Banaszak L J (1987) Structure of porcine heart cytoplasmic malate dehydrogenase combining X-ray diffraction and chemical sequence data in structural studies. Biochemistry USA 26, 2722–2734.

- Birktoft J J , Fu Z , Carnahan G E , Rhodes G , Roderick S L , Banaszak L J (1989a) Comparison of the molecular structure of cytoplasmic and mitochondrial malate dehydrogenase Biochem Soc Trans **17**, 301—304
- Birktoft J J , Rhodes G , Banaszak L J (1989b) Refined crystal structure of cytoplasmic malate dehydrogenase at 2.5 Å resolution Biochemistry USA **28**, 6065—6089
- Bleile D M , Schulz R A , Gregoxy E M , Harrison J H (1977) Investigation of the subunit interactions in malate dehydrogenase J Biol Chem **252**, 755—758
- Breiter D R , Resnik E , Banaszak L J (1994) Engineering the quaternary structure of an enzyme construction and analysis of monomeric form of malate dehydrogenase from *Escherichia coli* Protein Sci **3**, 2023—2032
- Cendrin F , Chroboczek J , Zaccai G , Eisenberg H , Mevarech M (1993) Cloning, sequencing and expression in *Escherichia coli* of the gene coding for malate dehydrogenase of the extremely halophilic archaebacterium *Haloarcula marismortui* Biochemistry USA **32**, 4308—4313
- Chien S M , Freeman K B (1984) Import of rat liver mitochondrial malate dehydrogenase Binding of the precursor to mitochondria, an intermediate step in import J Biol Chem **259**, 3337—3343
- Christian J H B , Walther J A (1962) Water relations of *Salmonella oranienburg*, stimulation of respiration by amino acids Biochim Biophys Acta **65**, 506—508
- Ciriacy M (1977) Isolation and characterization of yeast mutants defective in intermediary carbon metabolism and in carbon catabolite derepression Mol Gen Genet **154**, 213—220
- Clarke A R , Wiglez D B , Chia W N , Barstow D , Atkinson T , Holbrook J J (1986) Site-directed mutagenesis reveals role of mobile arginine residue in lactate dehydrogenase catalysis Nature **324**, 699—702
- Cooper T (1971) Activation of fatty acids in castor bean endosperm Biol Chem **246**, 3451—3455
- Cretin C , Luchetta P , Joly C , Decottignies P , Lepiniec L , Gadal P , Sallantin M , Huet J C , Pernollet J C (1990) Primary structure of sorghum malate dehydrogenase (NADP) deduced from cDNA sequence Homology with malate dehydrogenase (NAD) Eur J Biochem **192**, 299—303
- Curry R A , Ting I P (1973) Purification and crystallization of three isoenzymes of malate dehydrogenase from *Zea mays* seed Arch Biochem Biophys **158**, 213—224
- Decottignies P , Schmitter J M , Miginiac-Maslow M , Lemarechal P , Jacquot J P , Gadal P (1988) Primary structure of the light-dependent regulatory site of corn NADP-malate dehydrogenase J Biol Chem **263**, 11780—11785
- Donaldson R P , Fang T K (1987)  $\beta$ -oxidation and glyoxylate cycle coupled to NADH cytochrome c and ferricyanide reductases in glyoxysomes Plant Physiol **85**, 792—795
- Dry J B , Dimitriadis E , Ward A D , Wiskich J T (1987) The photorespiration hydrogen shuttle Synthesis of phthalonic acid and its use in the characterization of the malate/aspartate shuttle in pea (*Pisum sativum*) leaf mitochondria Biochem J **245**, 669—675
- Eisenberg H , Mevarech M , Zaccai G (1992) Biochemical, structural, and molecular genetic aspects of halophilism Adv Protein Chem **43**, 1—61
- Gietl C (1992) Malate dehydrogenase isoenzymes cellular locations and role in the flow of metabolites between the cytoplasm and cell organelles Biochem Biophys Acta

- 1100, 217—234
- Gleason W , Fu Z , Birtoft J , Banaszak L (1994) Refined crystal structure of mitochondrial malate dehydrogenase from porcine heart and the consensus structure for dicarboxylic acid oxidoreductases Biochemistry USA **33**, 2078—2088
- Gorisch H , Jany K (1989) Archaeabacterial malate dehydrogenase the amino-terminal sequence of the enzyme from *Sulfolobus acidocaldarius* is homologous to the eubacterial and eukaryotic malate dehydrogenase FEBS Lett **247**, 259—262
- Goward C R , Nicholls D J (1994) Malate dehydrogenase a model for structure, evolution and catalysis Protein Sci **3**, 1883—1888
- Grau U M , Trommer W E , Rossman M G (1981) Structure of the active ternary complex of pig heart lactate dehydrogenase with S-lac-NAD at 2.7 Å resolution J Mol Biol **151**, 289—307
- Hall M D , Levitt D G , Banaszak L J (1992) Crystal structure of *Escherichia coli* malate dehydrogenase A complex of the apoenzyme and citrate at 1.87 Å resolution J Mol Biol **226**, 867—882
- Harada K , Wolfe R G (1968) Malic dehydrogenase VII The catalytic mechanism and possible role of identical protein subunits J Biol Chem **243**, 4131—4137
- Hartl F U , Pfanner N , Nicholson D , Neupert W (1989) Mitochondrial protein import Biochim Biophys Acta **988**, 1—45
- Hatch M D , Slack C R (1969) NADP-specific malate dehydrogenase and glycerate kinase in leaves and evidence for their location in chloroplasts Biochem Biophys Res Commun **34**, 589—593
- Hecht K , Wrba A , Jaenicke R (1989) Catalytic properties of thermophilic lactate dehydrogenase and halophilic malate dehydrogenase at high temperature and low water activity Eur J Biochem **183**, 69—74
- Hill E , Tsernoglou D , Webb L , Banaszak L (1972) Polypeptide conformation of cytoplasmic malate dehydrogenase from an electron density map at 3.0 Angstrom resolution J Mol Biol **72**, 577—591
- Hochachka P W , Somero G N (1973) Strategies of Biochemical Adaptation, W B Saunders Co , p 358, Philadelphia, London, Toronto
- Hock B , Gietl C (1982) Cell-free synthesis of water-melon glyoxysomal malate dehydrogenase a comparison with the mitochondrial isoenzyme Ann NY Acad Sci **386**, 350—361
- Honka E , Fabry S , Niermann T , Palm P , Hensel R (1990) Properties and primary sequence of the L-malate dehydrogenase from the extremely thermophilic archaeabacterium *Methanothermus fervidus* Eur J Biochem **188**, 623—632
- Huang A H C (1987) The Biochemistry of Plants (Ed P K Stumpf) pp 91—119, Academic Press, London, New York
- Iijima S , Saiki T , Beppu T (1980) Physicochemical and catalytic properties of thermostable malate dehydrogenase from an extreme thermophile *Thermus flavus* AT-62 Biochim Biophys Acta **613**, 1—9
- Iijima S , Saiki T (1984) Reversible denaturation of thermophilic malate dehydrogenase by guanidine hydrochloride and acid J Biochem **95**, 1273—1281
- Issakidis E , Migniac-Maslow M , Decottignies P , Jacquot J-P , Cretin C , Gadal P (1992) Site-directed mutagenesis reveals the involvement of an additional thioredoxin-dependent regulatory site in the activation of recombinant sorghum leaf NADP-malate dehydrogenase J Biol Chem **26**, 21577—21583
- Issakidis E , Decottignies P , Migniac-Maslow M (1993) A thioredoxin-independent fully

- active NADP-malate dehydrogenase obtained by site-directed mutagenesis FEBS Lett **321**, 55—58
- Issakidis E , Saarinen M , Decottignies P , Jacquot J-P , Cretin C , Gadat P , Migniac-Maslow M (1994) Purification and characterization of the second regulatory disulfide site of recombinant sorghum leaf NADP-malate dehydrogenase J Biol Chem **269**, 3511—3517
- Issakidis E , Lemaire M , Decottignies P , Jacquot J-P , Migniac-Maslow M (1996) Direct evidence for the different roles of the N- and C-terminal regulatory disulfides of sorghum leaf NADP-malate dehydrogenase in its activation by reduced thioredoxin FEBS Lett **392**, 121—124
- Jaenicke R (1981) Enzymes under extremes of physical conditions Annu Rev Biophys Bioeng **10**, 1—67
- Jaenicke R (1988) Stability and self-organization of proteins Naturwissenschaften **75**, 604—610
- Joh T , Takeshima H , Tsuzuki T , Setoyama C , Shimada K , Tanase S , Kuramitsu S , Kagamiyama H , Morino Y (1987) Cloning and sequence analysis of cDNAs encoding mammalian cytosolic malate dehydrogenase Comparison of the amino acid sequences of mammalian and bacterial malate dehydrogenase J Biol Chem **262**, 15127—15131
- Johnson H P , Hatch M D (1970) Properties and regulation of leaf nicotinamide-adenine dinucleotide phosphate-malate dehydrogenase and “malic” enzyme in plants with the C4-dicarboxylic acid pathway of photosynthesis Biochem J **119**, 273—280
- Kelly C A , Nishiyama M , Ohnishi Y , Beppu T , Birktoft J J (1993) Determinants of protein thermostability observed in the 1.9 Å crystal structure of malate dehydrogenase from the thermophilic bacterium *Thermus flavus* Biochemistry USA **32**, 3913—3922
- Lemaire M , Migniac Maslow M , Decottignies P (1996) The catalytic site of chloroplastic NADP-dependent malate dehydrogenase contains a His/Asp pair Eur J Biochem **236**, 947—952
- Lorimer G H , Andrews T J (1980) The biochemistry of plants A comprehensive treatise In Photosynthesis (Eds M D Hatch, N K Boardman) pp 329—374, Academic Press, New York
- MacLachlan L , Haris P , Reid D , White J , Chapman D , Lucy J , Austen B (1994) A spectroscopic study of the mitochondrial transit peptide of rat malate dehydrogenase J Biochem **303**, 657—662
- McAlister-Henn L (1988) Evolutionary relationships among the malate dehydrogenases Trends Biochem Sci **13**, 178—181
- McAlister-Henn L , Blaber M , Bradshaw R , Nisco S (1987) Complete nucleotide sequence of the *Escherichia coli* gene encoding malate dehydrogenase Nucl Acids Res **15**, 4993
- Mernik N , Lewis R , Kollárová M , Mikulášová D , Kadlecová Z (1998) Characterization and Crystallisation of the Malate Dehydrogenase from *Streptomyces aureofaciens* Gen Physiol Biophys **17**, Suppl 1, 49—51
- Mettler I J , Beevers H (1980) Oxidation of NADH in glyoxysomes by a malate/aspartate shuttle Plant Physiol **66**, 555—560
- Metzler M C , Rothermel B A , Nelson T (1989) Maize NADP-malate dehydrogenase cDNA cloning, sequence and mRNA characterization Plant Mol Biol **12**, 713—722

- Míkulášová D , Kollárová M , Migniac-Maslow M , Decottignies P , Jacquot J-P , Kutejová E , Merník N , Egyudová I , Musrati R , Horecká T (1998) Purification and characterization of the malate dehydrogenase from *Streptomyces aureofaciens* FEMS Lett **159**, 299—305
- Mullinax T R , Mock J N , McEvily A J , Harrison J H (1982) Regulation of mitochondrial malate dehydrogenase J Biol Chem **257**, 13233—13239
- Muto S , Beevers H (1974) Lipase activities in castor bean endosperm during germination Plant Physiol **54**, 23—28
- Nicholls D J , Miller J , Scawen M D , Clarke A R , Holbrook J J , Atkinson T , Goward C R (1992) The importance of arginine 102 for the substrate specificity of *Escherichia coli* malate dehydrogenase Biochem Biophys Res Commun **189**, 1057—1062
- Nishiyama M , Matsubara N , Yamamoto K , Lijima S , Uozumi T , Beppu T (1986) Nucleotide sequence of the malate dehydrogenase gene of *Thermus flavus* and its mutation directing an increase in enzyme activity J Biol Chem **261**, 14178—14183
- Nishiyama M , Shimada K , Horinouchi S , Beppu T (1991) Role of threonine 190 in modulating the catalytic function of malate dehydrogenase from a thermophile *Thermus flavus* J Biol Chem **266**, 14294—14299
- Parker J H , Mattoon J R (1969) Mutants of yeast with altered oxidative energy metabolism selection and genetic characterization J Bacteriol **100**, 647—657
- Piontek K , Chakrabarti P , Schar H P , Rossman M G , Zuber H (1990) Structure determination and refinement of *Bacillus stearothermophilus* lactate dehydrogenase Proteins **7**, 74—92
- Poeydomenge O , Marolda M , Boudet A , Grima-Pettenati J (1995) Nucleotide sequence of a cDNA encoding mitochondrial malate dehydrogenase from eucalyptus Plant Physiol **107**, 1455—1456
- Pundak S , Eisenberg H (1981) Structure and activity of malate dehydrogenase from extreme halophilic bacteria of Dead Sea 1 Conformation and interaction with water and salt between 5M and 1M NaCl concentration Eur J Biochem **118**, 463—470
- Pundak S , Aloni H , Eisenberg H (1981) Structure and activity of malate dehydrogenase from the extreme halophilic bacteria of the Dead Sea 2 Inactivation, dissociation and unfolding at NaCl concentrations below 2M Salt, salt concentration and temperature dependence of enzyme stability Eur J Biochem **118**, 471—477
- Rao S T , Rossmann M G (1973) Comparison of super-secondary structures in proteins J Mol Biol **26**, 241—256
- Rehfeld D W , Tolbert N E (1972) Aminotransferases in peroxisomes from spinach leaves J Biol Chem **247**, 4803—4811
- Reng W , Riessland R , Scherbe R , Jaenicke R (1993) Cloning, site-specific mutagenesis expression and characterization of full-length chloroplast NADP-malate dehydrogenase from *Pisum sativum* Eur J Biochem **217**, 189—197
- Roderick S L , Banaszak L J (1986) The three-dimensional structure of porcine heart mitochondrial malate dehydrogenase at 3.0 Å resolution J Biol Chem **261**, 9461—9464
- Schatz G (1993) The protein import machinery of mitochondria Protein Sci **2**, 141—146
- Setoyama C , Ding S H , Choudhury B K , Joh T , Takeshima H , Tsuzuki T , Shmada K (1990) Regulatory regions of the mitochondrial and cytosolic isoenzyme genes participating in the malate-aspartate shuttle J Biol Chem **265**, 1293—1299

- Somero G N , Low P S (1976) Temperature a "shaping force" in protein evolution Biochem Soc Symp **41**, 33—42
- Sutherland P , McAlister -Henn L (1985) Isolation and expression of the *Escherichia coli* gene encoding malate dehydrogenase J Bacteriol **163**, 1074—1079
- Tzagoloff A , Akai A , Needleman R B (1975) Assembly of the mitochondrial membrane system isolation of nuclear and cytoplasmic mutants of *Saccharomyces cerevisiae* with specific defects in mitochondrial functions J Bacteriol **122**, 826—831
- Wigley D B , Gamblin S J , Turkenburg J P , Dodson E J , Piontek K , Murhead H , Holbrook J J (1992) Structure of a ternary complex of an allosteric lactate dehydrogenase from *Bacillus stearothermophilus* at 2.5 Å resolution J Mol Biol **223**, 317—335
- Wilks H M , Hart K N , Feeney R , Dunn C R , Murhead H , Chia W N , Barstow D A , Atkinson T , Clarke A R , Holbrook J J (1988) A specific, highly active malate dehydrogenase by redesign of a lactate dehydrogenase framework Science **242**, 1541—1544
- Wolosiuk R A , Schurmann P , Buchanan B B (1980) Thioredoxin and ferredoxin-thioredoxin reductase of spinach chloroplasts Methods Enzymol **69**, 382—391
- Wood D C , Hodges C T , Howell S M , Clary L G , Harrison J H (1981a) The N-ethylmaleimide-sensitive cysteine residue in the pH-dependent subunit interactions of malate dehydrogenase J Biol Chem **256**, 9895—9900
- Wood D C , Jurgensen S R , Geesin J C , Harrison J H (1981b) Subunit interactions in mitochondrial malate dehydrogenase Kinetics and mechanisms of reassociation J Biol Chem **256**, 2377—2382
- Yamazaki R K , Tolbert N E (1969) Malate dehydrogenase in leaf peroxisomes Biochem Biophys Acta **178**, 11—20
- Yancey P H , Clark M E , Hand S C , Bowlus R D , Somero G N (1982) Living with water stress evolution of osmolyte systems Science **217**, 1214—1222
- Yueh A Y , Chung C -S , Lai Y -K (1989) Purification and molecular properties of malate dehydrogenase from the marine diatom *Nitzschia alba* J Biochem **258**, 221—228
- Zaccaj G , Cendrin F , Haik Y , Borochov N , Eisenberg H (1989) Stabilization of halophilic malate dehydrogenase J Mol Biol **208**, 491—500

Final version accepted July 8, 1998