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Mitogen-Activated Protein Kinases and Their Role in Regulation of Cellular Processes

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Abstract. Mitogen-activated protein kinases (MAPKs) are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. The three major MAPK cascades are known, the extracellular signal-regulated protein kinase (ERK) cascade, c-Jun amino-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) cascade and p38-MAPK cascade. This paper is focused on characterization of these MAPK cascades in terms of their distribution and biological role in some pathological processes (apoptosis, hypertrophy) with a special orientation on the role of MAPKs in cardiovascular system during ischemia/reperfusion.

Key words: Mitogen-activated protein kinase — Ischemia — Kinase inhibitors

Introduction and characterization of MAPK cascades

Eukaryotic cells respond to extracellular stimuli by transmitting intracellular signals to coordinate appropriate responses. Protein phosphorylation is a major regulatory mechanism utilized by second messenger systems that are coupled to cell surface receptors or induced by cellular disturbances, such as stress stimuli and oncogenic transformation. Among the pathways that use protein phosphorylation to transduce these signals from the plasma membrane to nuclear and other cellular targets there are highly conserved mitogen-activated protein kinase (MAPK) cascades. These cascades are involved in the regulation of a wide variety of cellular processes such as proliferation, differentiation, development, cell cycle, cell death (Robinson and Cobb 1997; Chang and Karin 2001). These protein kinase cascades consist of a three-kinase module (Cobb and Goldsmith 1995) that includes:

- an activator of MEK (MEK kinase [MEKK] or MAPK kinase kinase),
- an activator of MAPK (MEK, MKK or MAPK kinase),
- the MAPK.

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Minireview

Malate Dehydrogenases – Structure and Function

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Abstract. Malate dehydrogenases (MDH, L-malate:NAD oxidoreductase, EC 1.1.1.37), catalyze the NAD/NADH-dependent interconversion of the substrates malate and oxaloacetate. This reaction plays a key part in the malate/aspartate shuttle across the mitochondrial membrane, and in the tricarboxylic acid cycle within the mitochondrial matrix. They are homodimeric molecules in most organisms, including all eukaryots and the most bacterial species. The enzymes share a common catalytic mechanism and their kinetic properties are similar, which demonstrates a high degree of structural similarity. The three-dimensional structures and elements essential for catalysis are conserved between mitochondrial and cytoplasmic forms of MDH in eukaryotic cells even though these isoenzymes are only marginally related at the level of primary structure.

Key words: Malate dehydrogenase — Enzyme mechanism — NAD/NADH

Introduction

Malate dehydrogenase (MDH) catalyzes the conversion of oxaloacetate and malate utilizing the NAD/NADH coenzyme system. MDH is a rather ubiquitous enzyme, for which several isoforms have been identified, differing in their subcellular localization and their specifity for the coenzyme NAD or NADP. In eukaryotic cells, at least two forms of the enzyme can be found. One isoform is a principal enzyme of the citric acid cycle operating within mitochondria. The other is found in the cytosol where it participates in the malate/aspartate shuttle. This shuttle exchanges reducing equivalent across the mitochondrial membranes in the form of malate/oxaloacetate rather than as NAD/NADH. A third isoenzyme was found in the glyoxysomes of yeast, where it converts malate produced from glyoxylate

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in the glyoxylate cycle (Minard and McAlister-Henn 1991). All MDHs are NAD-dependent except of the chloroplast enzyme which requires NADP as a coenzyme.

Primary and secondary structure

Malate dehydrogenases belong to the NAD-dependent dehydrogenases, which are one of the largest and best-studied families of nucleotide-binding proteins: over 100 different members have already been identified. In general, dehydrogenases are large protein molecules, e.g. lactate dehydrogenases (LDHs) or glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) are homotetramers, whereas MDHs or liver alcohol dehydrogenases (LADHs) are dimers. The lengths of their polypeptide chains vary slightly about 350 residues.

The amino acid sequences of MDHs show divergence into two main phylogenetic groups of closely related enzymes. MDHs from some eubacteria (e.g. *Escherichia coli*) have relatively high sequence identity with the mitochondrial enzymes of eukaryotes, whereas other eubacterial MDHs (e.g. *Thermus flavus*) are more closely related to the cytoplasmic and chloroplast enzymes (McAlister-Henn 1988; Goward et al. 1994). The evolution of MDHs, as deduced from amino acid sequence comparisons, fits well the pattern predicted by the endosymbiotic theory for the origin of mitochondria and chloroplasts (Fig. 1.). In the MDH family, the mitochondrial enzyme is more closely related to its prokaryotic counterpart than to the cytoplasmic MDH enzyme, implying a more recent divergence from a common ancestral gene (McAlister-Henn 1988). The extent of primary structure identity among pig mitochondrial MDH and pig cytoplasmic MDH is 19.6%. In comparison, among *Escherichia coli* MDH and pig mitochondrial MDH it is 58.3% and among *Escherichia coli* MDH and yeast mitochondrial MDH it is 47.9%. Greater similarities are found among functionally significant residues. 74% of residues involved in subunit interactions and 68% of those involved in coenzyme binding are identical.

The archaeal MDHs are very interesting, because this group has greater amino acid sequence identity with lactate dehydrogenases (LDH) comparing with other MDHs. This archaeal sequences suggest a link between the evolution of MDHs and LDHs. The link between LDHs and this LDH-like group of MDHs was reinforced by the crystallographic study of MDH from halophilic archaeon *Haloarcula marismortui* that showed a three dimensional structure similar to tetrameric LDL rather than to dimeric MDH (Richard et al. 2000).

Tertiary and quaternary structure

The three-dimensional structures of malate dehydrogenases from the cytoplasm and mitochondria of pig heart (Roderick and Banaszak 1986; Birktoft et al. 1989), eubacteria *Escherichia coli* (Hall et al. 1992) and Thermus flavus (Kelly et al. 1993), halophilic archaeon Haloarcula marismortui (Richard et al., 2000) and from chloroplast of plant Flaveria bidentis (Carr et al. 1999) have been determined at high resolution and it was found out that MDHs have homologous active sites, coenzyme binding site and quaternary structure. The comparison of the crystal

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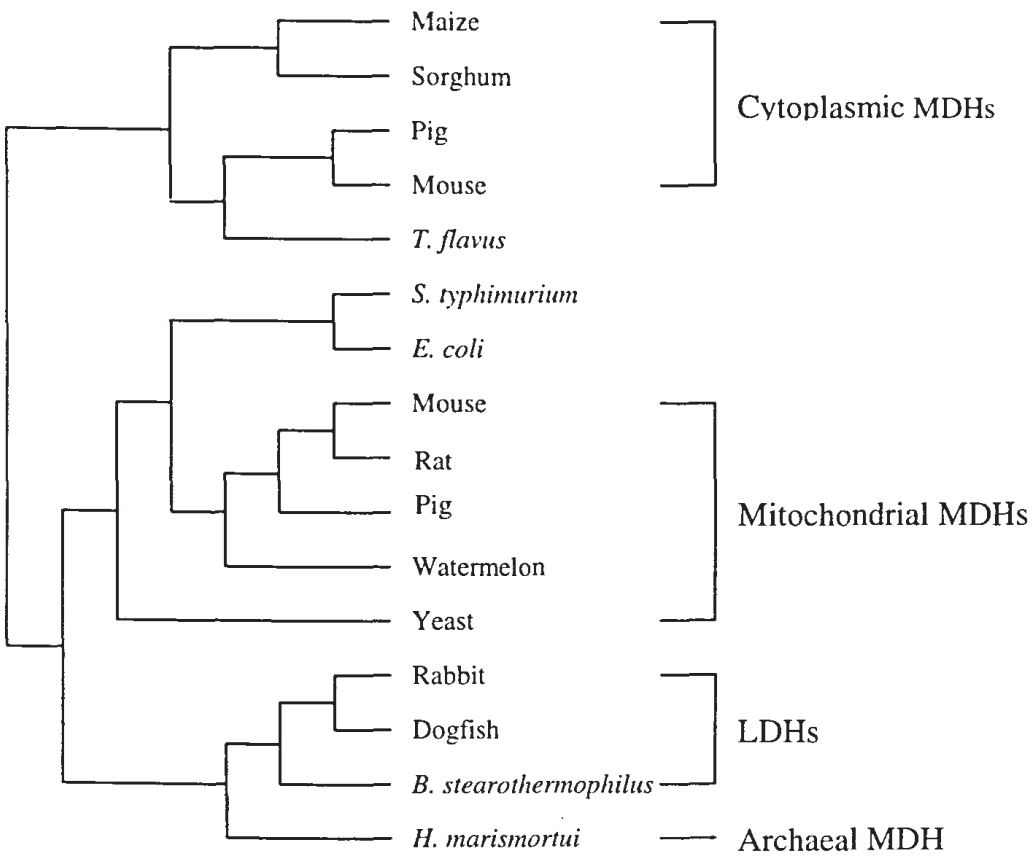

Figure 1. Phylogenetic tree of MDHs prepared by the program PILEUP (Goward and Nicholls 1994).

structure of Escherichia coli MDH-citrate complex with pig cytoplasmic MDH-NAD complex shows that the structures are essentially identical, although there is only about 20% sequence identity (Hall et al. 1992).

In general, MDHs are stable as dimers, which indicate the important connection of protein stability and their enzymatic activity. Each subunit contains two structurally and functionally distinct domains. The NAD-binding domain, occupying the amino-terminal half of each molecule, contains a parallel β-sheet structure (Rosman fold motif). The core dinucleotide binding structure is composed of four β-sheets and one α-helix. This NAD-binding domain is conserved to some extent in other dehydrogenases. The carboxy-terminal domain contains substrate binding site and amino acids that are necessary for catalysis. The active site of these enzymes is in a cleft between two domains (Hall et al. 1992).

Crystallographic studies have shown that the dimer interface consists mainly of interacting α-helices that fit compactly together. The active sites in these dimeric proteins are well separated from each other, the bound substrates are separated by about 30 Å (Breiter et al. 1994, Fig. 2).

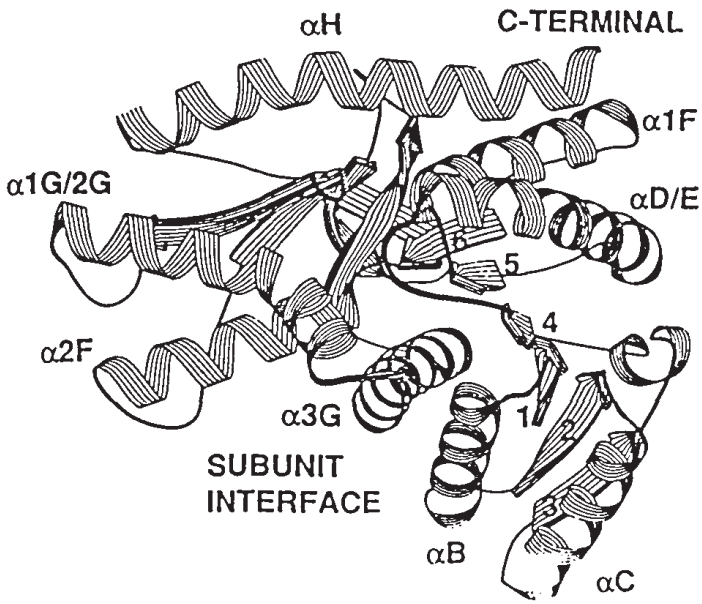

Figure 2. Three dimensional structure of monomer of MDH from *Escherichia coli* (Hall et al. 1992).

An approximate assessment of the stability of dimer can be obtained by examining the accessible surface area lost upon dimerization. For mitochondrial MDH, which is highly homologous with *Escherichia coli* MDH, this value is 13% and amounts to 3,200 $Å^2$ (Gleason et al. 1994). For *Escherichia coli* MDH, 3,250 $Å^2$ are lost upon dimerization, which is 13.5% of the monomer surface (Breiter et al. 1994). As is generally the case, the stability of the subunit-subunit interface in MDH is the result of direct hydrogen bonds, water-mediated hydrogen bonds and hydrophobic contacts.

Monomeric form of MDH from *Escherichia coli* was prepared by protein engineering (Breiter et al. 1994). Two of the highly conserved residues at the interface D45 and S226 were mutated to tyrosines to remove any columbic interactions and introduce a large bulky side chain. One of the two predicted sites, D45, correctly produced the desired monomeric form, the other, S226, did not. The double mutant was also a monomer, presumably because of the D45Y mutation. The specific activity of the altered enzymes was dependent on their oligomeric state. The dimeric mutant, S226Y, gave only small decreases in the specific activity. The monomeric D45Y enzyme had a specific activity that was dramatically decreased. The distance from D45 to the active site in its own subunit is greater than 20 Å and there is no obvious electrostatic network connecting the two regions. This work provides evidence for a catalytic mechanism that is largely dependent on the dimeric structure of the enzyme (Breiter et al. 1994).

The comparison of NADP-dependent MDH from chloroplast of plant *Flaveria bidentis* structure with that homologous NAD-dependent MDH structures shows how the light and redox regulation of an enzyme is achieved at the molecular level. NADP-MDH has amino-terminal and carboxy-terminal extensions when compared

to the NAD-MDH. Each of these extensions contain a disulfide bond. The disulfide bond at the carboxy-terminus causes the end of the polypeptide chain to turn and fold into the active site, thereby preventing access of the carboxy acid substrate, thus inactivating enzyme. This carboxy-terminal extension does not prevent coenzyme NADP binding, but it does interact strongly with the oxidized cofactor NADP. The role of the amino-terminal disulfide is less clear, but it may allow the amino-terminal extension to interact with the active site in the absence of the carboxy-terminal extension. The regulation *in vivo* is thought to occur through thiol-disulfide interchange of these terminal disulfide bonds with reduced thioredoxin. Thioredoxin is reduced in a reaction catalyzed by ferredoxin : thioredoxin reductase, by the reduced ferredoxin produced by the photochemical reaction of photosystem I (Carr et al 1999; Hirasawa et al. 2000).

The coemzyme specificity is given by residue 53, which is important for coenzyme binding and specificity of hydrogen bonding with the adenosine ribose hydroxyl groups. Residue 53 is conserved with an acidic side chain in all NAD-dependent MDHs, but is found as glycine in the NADP-dependent chloroplast MDH of maize and sorghum. The coenzyme specificity of MDH from *Thermus flavus* was altered from NAD to NADP by substitution of three residues on a loop including residue 53 (Nishiyama et al. 1993).

Catalytic mechanism

MDHs, similar to LDHs, catalyze the conversion of 2-hydroxy acids to the corresponding 2-keto acids (Banaszak and Bradshaw 1975; Birktoft et al. 1982), but MDHs are specific for oxaloacetate and LDH for pyruvate substrate. It is known from kinetic studies that the malate to oxaloacetate reaction is an ordered reaction with NAD/NADH binding first, followed by the dicarboxylic acid substrate (Silverstein et al. 1969). 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 (Nicholls et al. 1992; Goward and Nicholls 1994). The active-site contains a histidine/aspartate pair which forms a charge relay system, similar to that first observed in chymotrypsin, providing proton transfer, and an arginine whose guanidine unit plays the role of an anchor in binding the substrate carboxylate group (Lamzin et al. 1994) (Fig. 3). In majority of LDHs, the same position is occupied by an uncharged glutamine residue (Goward and Nicholls 1994). Site-directed mutagenesis was employed to modify Arg100 on the enzyme MDH to Gln (Cendrin et al. 1993; Golding and Dean 1998). This modification produced an enzyme that has considerably higher specifity for pyruvate (the substrate of LDH) than for oxaloacetate (the substrate of MDH) (Cendrin et al. 1993). From crystalographic studies of LDH and MDH complexes with substrate analogues and coenzymes, it has been suggested that discrimination between the two substrates

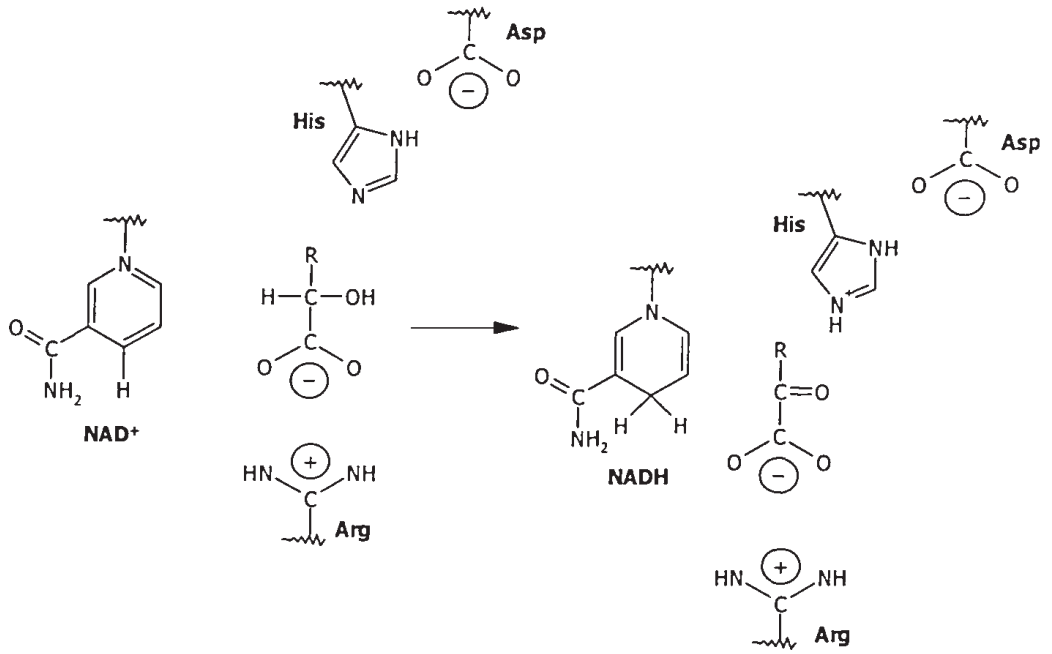

Figure 3. Reaction mechanism of MDH, scheme of NAD-dependent dehydrogenation of 2-hydroxy acids (Lamzin et al. 1994).

was the result of a charge imbalance inside the catalytic cavity (Wigley et al. 1992: Chapman et al. 1999).

In terms of catalytic mechanism, some MDHs have unique properties. Studies with mitochondrial MDHs have shown that this enzyme is allosterically regulated. High concentrations of malate stimulate the production of oxaloacetate, while high concentrations of oxaloacetate inhibit the reaction (Mullinax et al. 1982; Fahien et al. 1988). Citrate also affects MDH activity by very complex manner. It inhibits the reduction of oxaloacetate under all conditions. Citrate also inhibits malate oxidation, but only at low malate or NAD concentrations. When both malate and NAD concentrations are high (10 mmol/l and 5 mmol/l, respectively), citrate attempt to increase MDH activity (Gelpí et al. 1992). All three effectors (malate, oxaloacetate and citrate) bind to the same putative allosteric site (Mullinax et al. 1982).

Recent studies of malate dehydrogenases are focused into the nature of the inactivation processes and to explain whether or not exists active monomer of MDH. The oligomeric structure of MDH has a variety of biological implications. Some researches have suggested that the dimeric structure is critical for enzymatic activity. Two schools of thought arose surrounding the structure-function relationship. Harada and Wolfe (1968) first proposed the reciprocating compulsory ordered 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 corroborated by studies that showed a dramatic reduction of enzymatic ac-

tivity on dissociation to monomers at low enzyme concentration, at pH 5.0, and in the absence of substrates (Bleile et al. 1977; Wood et al. 1981a, 1981b). The second mechanism (Mullinax et al. 1982) introduces an equilibrium between 2 conformers of MDH, one of which preferentially binds the substrate analog – citrate and NAD, whereas the other binds NADH. This suggests an active monomer. Evidence for this idea is exhibited by an immobilized monomeric form of MDH (Jurgensen et al. 1981; DuVal et al. 1985), and a hybrid-modified form of MDH (Jurgensen and Harrison 1982; McEvily et al. 1985) having some catalytic function. The definitive answer appears to hinge on the question of whether a stable monomer can be isolated and whether it has activity.

Mitochondrial MDHs probably exist as a complex with fumarase and citrate synthase. The basis for complex formation is based on the fact that the standard free energy for the MDH catalyzed reaction is unfavorable in the direction of the operation of the citric acid cycle. Under standard conditions, the MDH reaction is favored in the direction of malate formation. It is believed that the formation of a citric acid cycle enzyme complex drives the cycle in the direction of oxalacetate formation by facilitating the transfer of oxaloacetate from MDH to citrate synthase (Beeckmans and Kanarek 1981; Robinson et al. 1987).

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