Molecular Analysis of the Malic Enzyme Gene (mae2)
of Schizosaccharomyces pombe

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Sequence analysis of a 4.6-kb HindIII fragment containing the malic enzyme gene (mae2) of Schizosaccharomyces pombe, revealed the presence of an open reading frame of 1695 nucleotides, coding for a 565 amino acid polypeptide. The mae2 gene is expressed constitutively and encodes a single mRNA transcript of 2.0 kb. The mae2 gene was mapped on chromosome III by chromoblotting. The coding region and inferred amino acid sequence showed significant homology with 12 malic enzyme genes and proteins from widely different origins. Eight highly homologous regions were found in these malic enzymes, suggesting that they contain functionally conserved amino acid sequences that are indispensable for activity of malic enzymes. Two of these regions have previously been reported to be NAD- and NADP-binding sites.

KEY WORDS — mae2; malic acid; wine; Schizosaccharomyces pombe.

INTRODUCTION

The malic enzyme catalyses the oxidative decarboxylation of malate to pyruvate and carbon dioxide in the presence of NAD(P)+ and divalent cations. The malic enzyme reaction is followed by successive decarboxylation and reduction of acetaldehyde to yield ethanol (Maconi et al., 1984). It allows for the continual turnover of the tricarboxylic acid (TCA) cycle when pyruvate is limiting (Hansen and Juni, 1975) and provides a means for the oxidation of TCA cycle intermediates. Three groups of malic enzymes, depending on coenzyme specificity and ability to catalyse the decarboxylation of oxalacetate, have been reported (Figure 1). L-Malate: NAD+ oxidoreductase (oxalacetate-decarboxylating; EC 1.1.1.38) uses NAD+ as coenzyme, and has been found in lactobacilli (Korkes et al., 1950), streptococci (London and Meyer, 1969), Escherichia coli (Murai et al., 1972; Yamaguchi et al., 1973) and Schizosaccharomyces pombe (Temperli et al., 1965). L-Malate: NAD+ oxidoreductase (decarboxylating; EC 1.1.1.39) is the malic enzyme most commonly found in the mitochondria of plants and animals (Artus and Edward, 1985). It has also been isolated from Ascaris lumbricoides (Saz and Hubbard, 1957), E. coli (Murai et al., 1972) and Pseudomonas diminuta (Suye et al., 1992). This enzyme can use NADP+ as a coenzyme in some cases, but prefers NAD+. L-Malate: NADP+ oxidoreductase (oxalacetate-decarboxylating; EC 1.1.1.40) is NADP+-dependent and has been found in plants (Rothermel and Nelson, 1989), animals (Bagchi et al., 1987), Aspergillus nidulans (McCullough and Roberts, 1974) and Neurospora crassa (Zink, 1967). Although most NADP+-dependent malic enzymes can also use NAD+, NADP+ is preferred and acts as a non-competitive inhibitor of NAD+ activity in these enzymes (Hatch and Mau, 1977).

The S. pombe malic enzyme has a pH optimum of 3.5–4.0 (Osothipil and Subden, 1986a; Young, 1973) and cultures are able to metabolize up to 20 g/l malic acid (De Queiroz and Pareilleux, 1990; Taillandier et al., 1988), probably due to a very high substrate affinity (Temperli et al., 1965). The low $K_m$ of 3.2 mM for the S. pombe malic enzyme is in contrast with the $K_m$ value of 50 mM for Saccharomyces cerevisiae (Fuck et al., 1973), and 15 mM for Saccharomyces bailii (Kuczynski and Radler, 1982).

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Taillardier and Strehaiano (1991) showed that malic acid is not integrated into biomass after its catabolism by *S. pombe*. It is completely metabolized to ethanol and CO₂ during anaerobiosis, and to CO₂ under aerobiosis (Mayer and Temperli, 1963). Malic acid degradation is glucose-dependent (Magyar and Panyik, 1989) under both aerobic and anaerobic conditions (De Queiros and Pareilleux, 1990), suggesting that the metabolism of malate requires energy, supposedly for transport of malic acid into the cell (Taillardier and Strehaiano, 1991).

Mutants of *S. pombe* strain 972h⁻ defective in malic acid utilization (mau⁻ mutants) were isolated by Osothsilp and Subden (1986a). The *S. pombe* malic enzyme gene was mapped 29 map units from the ade6 gene on the right arm of chromosome III near the centromere (Osothsilp, 1987).

Osothsilp and Subden (1986a) reported the involvement of two additional enzymes, malate dehydrogenase and malate permease, in the metabolism of malate. Malate dehydrogenase (EC 1.1.1.37) mediates the conversion of l-malate into oxalacetate. Under anaerobic conditions, the malic enzyme alone is capable of supporting malate metabolism, although not as efficiently as in the presence of malate dehydrogenase (Osothsilp, 1987). During aerobiosis both the malic enzyme and malate dehydrogenase are required for malate utilization. The malate permease gene is constitutively expressed (Osothsilp and Subden, 1986b) and is inhibited by inhibitors of oxidative phosphorylation. The permease plays an important role in the active transport of malate.

The purpose of this study was to determine the DNA sequence of the mau2 gene, analyse the molecular structure of the gene, and determine the size of the mRNA transcript. Furthermore, the DNA and amino acid sequences of the *S. pombe* malic enzyme were compared with those of other known malic enzymes. Several conserved regions were found in the different malic enzymes, thereby implicating possible sites involved in the malic enzyme activity.

**MATERIALS AND METHODS**

**Strains and media**

*Schizosaccharomyces pombe* 972 leu1-32 h⁻ (wild type) and *S. pombe* 972 leu1-32 me⁻ h⁻ LH-67 (mae2⁻ mutant) were used in this study. *S. pombe* cells were grown in YEPD medium (1% yeast extract, 2% bactopeptone, 2% glucose), supplemented with 0-8% l-malate (Sigma, St Louis, MO) when required. Transformants were selected on YNB (0-17% yeast nitrogen base without amino acids [Difco Laboratories, Detroit, MI], 0-5% (NH₄)₂SO₄, 2% glucose, 1-7% bacto-agar
[Difco Laboratories, Detroit, MI] and malate-glucose indicator agar (MGIA), described previously by Osothsilp and Subden (1986a). E. coli HB101 (hisA104 leuB6 supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 mtl-1 recA13 mcrB) was grown in TY medium (1% yeast extract, 1% NaCl, 2% tryptone) with ampicillin for plasmid preparations and in Luria Broth (0.5% yeast extract, 1% NaCl, 1% tryptone) for transformation purposes.

Yeast transformation

The S. pombe mae2 mutant strain was cultured in YEPD and competent cells were prepared by means of the lithium acetate procedure (Moreno et al., 1991). Transformants were selected on YNB plates (4 days at 30°C) and then plated on MGIA to verify their ability to degrade malic acid.

Malic acid test

S. pombe wild type, mae2−, and mae2− transformed with pMV651 and pMV652, were inoculated in 10 ml YEPD containing 0.8% L-malate (final concentration 5 × 10−6 cells/ml), and incubated on a gyrotory shaker at 30°C for 4 days. The cells were harvested by centrifugation and the supernatant was filter-sterilized with Millex-GS 0.22 μm filter units (Millipore, Bedford, MA). The L-malic acid content of the supernatant was determined with the L-malic acid test kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturers’ instructions.

Pulse-field gel electrophoresis and Southern blotting

S. pombe 972 (wild type) was grown in 100 ml YEPD until the late logarithmic phase. Chromosomal DNA plugs were prepared and the chromosomes separated by contour-clamped homogeneous electric field (CHEF) electrophoresis as described by van der Westhuizen and Pretorius (1992), with some modifications. The DNA was loaded in a 0.75% agarose (Pulsed Field Certified, Bio-Rad Laboratories, Hercules, CA) gel, and run in 0.5 × TBE buffer for 150 h at 50 V and 14°C, with a switching time of 60 min.

Standard procedures (Sambrook et al., 1989) were used to prepare the gel for Southern blotting and to transfer the DNA to a 0.45 μm Hybond-N nylon membrane (Amersham International plc, Buckinghamshire, U.K.) as described by Sambrook et al. (1989). The internal 610-bp EcoRV/BanII [α-32P] DNA fragment of mae2 was used as a probe for the detection of the mae2 mRNA transcript.

RNA isolation and northern analysis

S. pombe mae2− transformed with pMV652, was cultured in 10 ml YEPD at 30°C for 48 h. Total RNA was isolated as described by Laing and Pretorius (1992), with the exception that the cells were vortexed for only 30 s. 30 μg of total RNA were separated in a 0.8% agarose/2.2 M-formaldehyde denaturing gel and transferred to a 0.45 μm Hybond-N nylon membrane (Amersham International plc, Buckinghamshire, U.K.) as described by Sambrook et al. (1989). The internal 610-bp EcoRV/BanII DNA fragment of mae2 was used as a probe for the detection of the mae2 mRNA transcript.

Nucleotide sequence accession number

The sequence of the S. pombe mae2 gene is available in GenBank under accession number U00621.
RESULTS AND DISCUSSION

Utilization of malic acid by yeast transformants

*Schizosaccharomyces pombe* mae2" transformed with pMV651 and pMV652 gave rise to blue-green colonies on MGIA plates after an incubation period of 4 days at 30°C. Osothsilp and Subden (1986a) reported that the blue-green colour was due to a pH shift from 3.3 to approximately 5.0 during the degradation of malate. The wild-type and transformed strains degraded more than 99.7% of the malic acid present, whereas the mutant strain was unable to utilize malic acid.

Chromosomal localization of the mae2 gene and size of mRNA transcript

Osothsilp (1987) mapped the mae2 gene 29 map units from the ade6 gene on chromosome III of *S. pombe*. Southern analysis of the CHEF gel confirmed that mae2 is located on chromosome III (Figure 2). The mae2 gene encodes a single mRNA transcript of approximately 2.0 kb (Figure 3), which is in the range of 1.67-4.0 kb for duck liver malic enzyme (Hsu et al., 1992) to 2.264 kb for the *Ascaris suum* malic enzyme (Kulkarni et al., 1993).

Nucleotide sequence of the mae2 gene

A restriction map of the mae2 gene is shown in Figure 4. The 4.6-kb HindIII fragment contains an open reading frame (ORF) of 1695 bp, with the ATG at nucleotide (nt) 2184 and the stop codon at nt 3879. The DNA sequence of the ORF and 3'-flanking region, as well as the inferred amino acid sequence, are shown in Figure 5. The 5'-flanking sequences of mae2 contain several TATA-like sequences (data not shown).

The genetic structure of *S. pombe* differs in many aspects from that of *S. cerevisiae*, e.g. codon usage, organization of ribosomal RNA genes, presence or absence of introns, intron splicing recognition sequences, and polyadenylation signal sequences (Barker et al., 1987; Nischt et al., 1986; Riva et al., 1982; Simon-Becam et al., 1978). The intron and splicing apparatus and use of transcriptional and translational signals of *S. pombe* display a closer resemblance with that of mammalian cells than with *S. cerevisiae* (Jones et al., 1988; Kraifer et al., 1985; Mertens and Gallwitz, 1987).

The presence of a poly(A) tail in eukaryotic mRNA increases the cytoplasmic stability of mRNA (Bernstein and Ross, 1989) and enhances the efficiency of its translation (Munroe and Jacobson, 1990). By using different poly(A) sites on the primary transcript, the size and levels of particular mRNAs can be varied (Smith et al., 1989) as is the case for the murine malic enzyme gene (Bagchi et al., 1987) and rat malic enzyme gene (Morioka et al., 1989). Three signal motifs comprise a functional poly(A) site on mRNAs in mammalian cells (Manley, 1988): a highly conserved AAUAAA occurs upstream of the poly(A) site, a CA or UA usually forms the cleavage site, and a more divergent G/U- or U-rich element is located downstream. The conserved AAATAAA
sequence was found 318 bp, 344 bp, 406 bp and 489 bp downstream of the mae2 gene. The AUUAAA motif, present in approximately 10% of eukaryotic mRNAs (Wickens and Stephenson, 1984), is present 260 bp and 494 bp downstream of mae2, overlapping with the AATAAA motif located 489 bp 3'-flanking to the mae2 gene. T-rich areas are present downstream of the AATAAA motif (406 bp) and the ATTAAA motif (206 bp).

The AATAAA motif found in mammalian genes plays a small role, if any, in the polyadenylation of S. cerevisiae mRNA (Hyman et al., 1991). Instead, S. cerevisiae uses a TAG...TATGTA or a TATAA motif at a variable distance (14 to 1240 nt) upstream of the poly(A) site (Abe et al., 1990; Russo et al., 1991). Humphrey et al. (1991) reported that the mechanism for mRNA 3'-end formation in S. pombe is closely related to that of S. cerevisiae. The S. cerevisiae motifs are also present in the 3'-flanking sequences of mae2. TATAA motifs were found 253 bp and 444 bp downstream of mae2, whilst a TATGTA motif is located 380 bp downstream of mae2.

Comparison of amino acid sequences of 13 different malic enzymes

The molecular structures of the malic enzymes of human mitochondria, mouse, rat, duck liver, Zea mays, Populus deltoides, Flaveria trinervia, Flaveria linearis, Mesembryanthemum crystallinum and A. saum (Table 1) have been reported. Comparison of the S. pombe mae2 with these malic enzyme genes showed a DNA identity as high as 52%. There is also a high amount of identity on the amino acid level, e.g. 36-6% over 555 amino acids for the human malic enzyme. The GCG Genbank search also revealed homology with an E. coli sequence, namely 'E. coli sbeA', a translational fusion protein of part recA with another gene 140 kb distant' (Mahajan et al., 1990). The high amount of identity obtained with this sequence (Table 1) indicates that it may contain the coding region for E. coli malic enzyme. There is also a high amount of identity with the Phaseolus vulgaris (bean) cinnamyl-alcohol dehydrogenase (CAD4) gene (Walter et al., 1988), later reported (Walter et al., 1990) to be more likely a malic enzyme.

We compared the amino acid sequences of the different malic enzymes and found several regions of highly conserved amino acid sequences. The number of amino acids between each set of elements was also consistent (Figure 6).

Wierenga et al. (1986) proposed a consensus sequence of h-G-G-G-n-n-n-n-n-n which serves as a ββ fold for the binding of ADP-moieties. It has a characteristic arrangement of glycines (G), non-polar (n) and hydrophilic (h) residues at certain crucial positions on a compact ββ fold. According to Loeber et al.
Figure 5. DNA and amino acid sequence of the coding and 3'-flanking region of mae2. The deduced amino acid sequence of the protein is written below the nucleotide sequence. The numbers on the left refer to the nucleotide sequence, and those on the right refer to the protein sequence.
Table 1. DNA and amino acid homology of the *S. pombe* malic enzyme with other known malic enzymes

<table>
<thead>
<tr>
<th>Source</th>
<th>DNA % identity (bp)</th>
<th>Amino acid % identity (aa)</th>
<th>EC number</th>
<th>GCG Accession code</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human mitochondria</td>
<td>52.4 (1532)*</td>
<td>36.6 (555)†</td>
<td>40</td>
<td>M55905</td>
<td>Loeber et al. (1991)</td>
</tr>
<tr>
<td>Murine ME</td>
<td>51.9 (958)</td>
<td>35.0 (554)</td>
<td>40</td>
<td>J02652</td>
<td>Bagchi et al. (1987)</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td>40</td>
<td>M30596</td>
<td>Magnuson et al. (1986)</td>
</tr>
<tr>
<td>Duck liver</td>
<td></td>
<td></td>
<td>40</td>
<td>S38982</td>
<td>Hsu et al. (1992)</td>
</tr>
<tr>
<td>Zea maysa</td>
<td>52.9 (991)</td>
<td>35.0 (552)</td>
<td>40</td>
<td>J05130</td>
<td>Rothermel and Nelson (1989)</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>52.1 (1479)</td>
<td>34.7 (552)</td>
<td>40</td>
<td>J03825</td>
<td>Walter et al. (1988)</td>
</tr>
<tr>
<td><em>P. deltoides</em></td>
<td></td>
<td></td>
<td>40</td>
<td>X56233</td>
<td>Van Doorselaere et al. (1991)</td>
</tr>
<tr>
<td><em>F. trinervia</em></td>
<td>50.9 (1033)</td>
<td>33.0 (552)</td>
<td>40</td>
<td>X57142</td>
<td>Börsh and Westhoff (1990)</td>
</tr>
<tr>
<td><em>F. linearis</em></td>
<td></td>
<td></td>
<td>40</td>
<td>M59415</td>
<td>Rajeevan et al. (1991)</td>
</tr>
<tr>
<td><em>A. suum</em></td>
<td>33.1 (356)</td>
<td></td>
<td>38</td>
<td>M81055</td>
<td>Kulkarni et al. (1993)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>34.8 (561)</td>
<td></td>
<td>38/40*</td>
<td>X55956</td>
<td>Mahajan et al. (1990)</td>
</tr>
<tr>
<td><em>Mesembryanthemum</em></td>
<td>41.0 (442)</td>
<td></td>
<td>40</td>
<td>S44544</td>
<td>Cushman (1992)</td>
</tr>
</tbody>
</table>

*52.4% identity over 1532 bp etc.
†36.6% identity over 555 amino acids etc.
‡EC 1.1.1.38/39/40, classified according to cofactor specificity.
§GCG Genbank accession code.
*Uncertain whether it represents the NAD- or NADP-linked enzyme.

(1991), NAD-binding sites display the consensus $G - G - G - G - G - G - G - G - n - n - (n, neutral; b, basic; a, acidic), whereas the NADP-binding sites have the sequence $G - G - A - A - G - G - A - n b - n b - n b - (n, neutral; b, basic; a, acidic)$ (Hanukoglu and Gutfinger, 1989; Scrutton et al., 1990). Replacement of the two alanine residues (underlined above) with glycine residues converted the NADP-specificity of a glutathione reductase to a NAD-specificity (Scrutton et al., 1990).

A consensus LGLGDLG sequence was reported for the NAD-binding site in malic enzymes (Hsu et al., 1992; Wierenga et al., 1986), in agreement with the $X - Y - Y - X - Gly - X - Gly - Y - X - Gly$ (X is a hydrophobic residue, Y is variable, and the indicated spacing of the three Gly residues is retained) reported for different NAD-linked dehydrogenases (Rossman et al., 1975). The present study, however, revealed an expanded consensus sequence of ILGLGD - G - G for the NAD-binding sites in malic enzymes (region B in Figure 6). However, *S. pombe* has an isoleucine instead of the second leucine residue.

A GAGEAGTGIA consensus for a NAD-binding site in malic enzymes was reported by Rothermel and Nelson (1989) and Börsh and Westhoff (1990). The sequences of maize, *P. vulgaris*, *P. deltoides*, *F. trinervia* and *Mesembryanthemum* display a GAGEAGTGIA sequence for region E (Figure 6), whereas *S. pombe* and *E. coli* have a closely related sequence (GAG - AG - GIA). The human, murine, rat and duck malic enzymes have the consensus GAGEAALGIA, in agreement with the proposed NADP site of Scrutton et al. (1990). The sequences of *A. suum* and *F. linearis* do not fit either of the reported consensus sequences, but possess some characteristics of both. All the malic enzymes reported in Figure 6, except *F. linearis*, have the GAG - A - GIA sequence for region E. Furthermore, all the EC 1.1.1.40 malic enzymes (except *F. linearis*) display the consensus GAGEA - GIA. The consensus glutamate residue (E) might therefore be important for the coenzyme specificity of the malic enzyme. However, it is difficult to designate a physiological importance to the presence of the glutamate residue in the NADP-linked enzymes without further investigation. It is clear that the consensus sequences for the NADP-binding sites suggested by Scrutton et al. (1990) do not apply for these sites in malic enzymes described to date.

For region A, a consensus amino acid sequence of P-VYTPVQ-AC was found, with slight variations for *S. pombe*, *Mesembryanthemum* and *E. coli*. This region does not show significant identity with any regions in proteins other than the
**Consortium**

- **S. pombe**
- **Human**
- **Murine**
- **Duck**
- **Maize**
- **P. vulgaris**
- **P. del.**
- **F. trin.**
- **F. lin.**
- **Mesembr.**
- **A. suum**
- **E. coli**

**Consensus**

- **ILGLDLGGCG**
- **Q**
- **Q**
- **AY**
- **IN**
- **VTV**
- **DI**
- **ECT**
- **T**
- **T**

**Region A**

- **97**
- **138**
- **98**
- **95**
- **89**
- **216**
- **141**
- **183**
- **202**
- **155**
- **158**
- **257**

**Region B**

- **160**
- **195**
- **158**
- **155**
- **145**
- **276**
- **201**
- **243**
- **462**
- **215**
- **220**
- **318**

**Region C**

- **220**
- **222**
- **228**
- **184**
- **178**
- **230**
- **234**
- **272**
- **229**
- **224**
- **228**
- **235**

**Region D**

- **193**
- **228**
- **202**
- **189**
- **185**
- **200**
- **245**
- **287**
- **296**
- **259**
- **256**
- **361**

**Region E**

- **302**
- **339**
- **298**
- **288**
- **419**
- **344**
- **806**
- **92**
- **358**
- **363**
- **459**

**Region F**

- **362**
- **413**
- **370**
- **360**
- **493**
- **418**
- **460**
- **186**
- **432**
- **435**
- **540**

**Region G**

- **342**
- **441**
- **396**
- **386**
- **520**
- **446**
- **488**
- **508**
- **519**
- **463**
- **568**

**Region H**

- **409**
- **440**
- **422**
- **428**
- **425**
- **425**
- **548**
- **473**
- **515**
- **527**
- **490**

**Consensus**

- **FLGAGAAGTIAR**
- **3FQATAVN**
- **VR**
- **A**
- **IKPTVLIQGQAGCTFTK**
- **VR**
- **SIIL**
- **G**
- **Q**
- **S**
- **Q**

Figure 6. Regions of conserved amino acid sequences observed in the different malic enzymes. The numbers of amino acids between the regions are indicated. Bold letters indicate conserved residues and replacements are listed below the consensus sequence. The amino acid positions given here are as they appear in the GCG database, and do not therefore necessarily correspond with those published. Abbreviations used are: *P. vulgaris* (*P. vulgaris*), *P. del.* (*P. deltoides*), *F. trin.* (*F. trinervia*), *F. lin.* (*F. linearis*), *Mesembr.* (*Mesembryanthemum*), *B. stearo.* (*B. steareomorphus*).
malic enzymes in Table 1. Rothermel and Nelson (1989) reported a VYPTVGSEA sequence at position 221–129 on the maize malic enzyme, that showed homology with the proposed NADP-binding site (VPTTVGSA) of the enoyl reductase domain of goose fatty acid synthetase, and also had some identity with the VPTMEKA region of human glyceraldehyde-3-phosphate dehydrogenase, an NADP-dependent enzyme.

Hsu et al. (1992) reported that 'Cys 99' in duck liver malic enzyme (corresponding to S. pombe's position 111 in region A) was important for the binding of L-malate and divalent metal ions. Modification of a thiol group at the malate-binding site of the NADP-linked malic enzyme from A. suum resulted in a decreased binding of malate (Gavva et al., 1991). Drincoovich et al. (1992) found evidence for the presence of two sulphhydryl groups, at positions 230 and 284, located at or near the active site of the maize malic enzyme. The cysteine at position 230 corresponds to position 111 (therefore the Cys 99 in duck liver) of the S. pombe amino acid sequence. This cysteine is conserved in all of the malic enzymes in Figure 6, except for S. pombe, which contains an isoleucine instead of the cysteine residue. The other cysteine, found at position 284 in the maize malic enzyme, is present in all the NADP-dependent malic enzymes (Figure 6, region B).

Hsu et al. (1992) suggested that Cys 99 is involved in binding of malate and divalent ions in duck liver malic enzyme, whilst Drincoovich and Andreo (1992) reported this cysteine to be adjacent to the NADP-binding site in maize malic enzyme. It therefore seems that there is uncertainty whether the sequence of region A constitutes an NADP site or the malate-binding site. However, the order of events, as reported by Pry and Hsu (1980) for pigeon liver malic enzyme, could explain this contradiction. They found that Mn²⁺ binds first, followed by NAD(P) and malate. Cys 99 could be in contact distance from the bound divalent metal ion (Schimerlik and Cleland, 1977) and could therefore play a role in the formation of a Cys-Mn²⁺ ligand. Kieck et al. (1984) found that the sulphhydryl group in A. suum is not essential for enzyme activity, but the reactivity of the thiol group is modulated by the divalent metal ion. The metal ion presumably polarizes the carboxyl group of the oxalacetate intermediate during decarboxylation (Gavva et al., 1991), in agreement with the accepted theory for metal-assisted decarboxylation of β-keto carboxylic acids (Grissom and Cleland, 1988). The Cys-Mn²⁺ ligand therefore seems to play an important role in the catalytic mechanism; however, the absence of a 'Cys 99' in S. pombe malic enzyme questions the requirement for this residue.

No significant homology with amino acid sequences in the Genbank database was found for regions C, D, F, G and H, and the physiological importance of these regions remains unknown. However, the fact that these conserved regions were found consistently in the malic enzymes from bacteria, fungi, nematodes, higher plants, animals and humans, suggests that these regions are indispensable for the expression of malic enzyme activity.

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