The Kinetic Characteristics of Malic Enzyme in Human Breast Tissue Cancer Cell Lines MCF-7 and MDA-MB-231


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Abstract

Background: A high level of replication is one of the main indicators of tumors. Tumor cells have to manufacture and transport macromolecules into daughter cells. One of the required enzymes is malic enzyme, which generates the NADPH for fatty acid synthesis in order to make cell membrane and pyruvate, and support the glycolysis pathway to supply the energy demand. Due to the enormous proliferation of cancer cells, it is likely that the activity of malic enzyme in cancer cells is more than normal cells. The aim of this study is to survey the kinetics of malic enzyme in tumor and normal breast tissues.

Methods: We obtained the tumor and normal breast tissue specimens directly from the operating room. The assays were performed with partially purified samples under optimum conditions for the substrate and co-factor requirements. The velocity of the enzyme or Michaelis-Menten constant, maximum velocity, and the amount of inhibitor that reduced the enzyme activity by 50% were obtained and calculated in all samples.

Results: The Michaelis-Menten constant for malate was lower in tumors compared to normal samples. In contrast, the maximum velocity for malate in tumors was higher than normal tissues, whereas the amount of inhibitor that reduced the enzyme activity by 50% of guanidine hydrochloride and sodium chloride were both higher in tumors than normal tissues.

Conclusion: The obtained results indicated that the malic enzyme kinetics had different patterns in tumor tissues in comparison with normal tissues. A higher affinity of malic enzyme for pyruvate production in tumors supported high aerobic glycolysis. Moreover, it could be an approach to connect glutaminolysis to the glycolysis pathway. Malic enzyme could be a target to inhibit the glycolysis and glutaminolysis pathways in tumors.

Keywords: Malic enzyme, Breast cancer, Kinetics, Cancer metabolism
Introduction

Excessive growth and proliferation are the most important features of cancer cells. These cells need energy and cellular components such as a cell membrane, nucleotides, and proteins to grow and proliferate. In order to meet these requirements, cancer cells use various metabolic approaches. High aerobic glycolysis, glutaminolysis, and substantial fatty acid synthesis are the most well-known characteristics of cancer cell metabolism.1 Some essential metabolites and cofactors are necessary to support the current pathways. Pyruvate, NAD, and NADPH have key roles in supporting the advancement of glycolysis and fatty acid synthesis, respectively.2 Malic enzyme (ME) is an important enzyme in metabolism that catalyzes the interconversion of malate to pyruvate with the concomitant regeneration of NADPH.3 Malic enzyme, by producing pyruvate and NADPH, provides abundant vital resources for glycolysis and fatty acid synthesis.4 Interestingly, most studies indicate that ME expression is significantly up-regulated in different types of human cancers.5

The environmental parameters of pH, oxygen, and nutrient availability use different approaches to influence enzyme kinetics such as ME.6 The tumor micro-environment has a very heterogeneous oxygen pressure, pH, and availability of other metabolites. The nature and importance of tumor heterogeneity has been emphasized in enzyme studies because of the use of cell culture that have a normal range of pH without any fluctuations, and an excess of oxygen and nutrients.7-9

Both the MCF-7 and MDA-MB-231 breast cancer cell lines are of interest because their metabolism pattern completely differ from normal cells. Malic enzyme has a specific role in metabolism. Hence, we can compare the features of ME in two cell lines that have different metabolic patterns.8 The aim of the current study is to elucidate the kinetic parameters of ME in human breast cancer samples by considering the important role of ME in cancer metabolism and the strong effect of the micro-environment on the enzyme’s kinetics with attention to different characteristics of the tumor milieu.

Materials and Methods

Clinical sample collection

We obtained 10 human breast tumor samples from Apadana Hospital, Ahvaz, Iran. The samples were taken during the mastectomy procedure. Control samples comprised normal tissues located away from the tumor site. The samples were immediately preserved in liquid nitrogen, transported to the laboratory, and stored at -80°C. Two independent expert pathologists from the Pathology Laboratory of Apadana Hospital carried out the pathological examinations of the tumor tissues. The Ethics Committee of Ahvaz Jundishapur University of Medical Sciences approved this study, which was conducted according to the Guide for Human Study by the National Academy of Sciences (National Institutes of Health). All patients provided informed consent for study participation.

Sample preparation and ME partial purification

The frozen tumor and normal tissues were homogenized at 1:5 (w:v) in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 10% v:v glycerol, 1 mM EDTA, 1 mM EGTA, 10 mM β-2 ME). At the time of homogenization, we added a few crystals of phenylmethylsulfonyl fluoride (PMSF). The samples were homogenized with a homogenizer (Miccra, Germany) and centrifuged for 30 min at 25000 g at 4°C. The supernatant was decanted and held on ice until use. The low molecular weight metabolites and ions were removed from the supernatant by Sephadex G-25 columns (1×5 cm; Sigma, Germany), and equilibrated in a homogenizing buffer.

According to the cytosolic ME pI, partial purification of ME was done with the preparation of a DEAE-Sephadex column (1×10 cm), which was equilibrated in 20 mM Tris-HCl (pH 7.5) assay buffer. Following equilibration, approximately 2 mL of the crude extract was placed at the top of the column and the column
was washed with 30 mL of the assay buffer to remove any unbound proteins, such as malate dehydrogenase (MDH) and lactate dehydrogenase (LDH). Afterwards, we applied a linear salt gradient (0-2 M KCl) to the column to elute the ME. The high-activity fractions were pooled and held at 4°C until use.9 This sample was used for the subsequent kinetic characterization of ME.

Cell culture and cytosolic fraction preparation

We obtained the MCF-7 and MDA-MB 231 cells from Pasteur Institute’s collection of cell cultures (Tehran, Iran). The cells were maintained at 37°C and 5% CO₂ in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco, Life Technologies, USA). Before each experiment, we removed the culture medium and washed the plated MCF-7 and MDA-MB 231 cells with PBS medium that contained 138 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 15 mM KH₂PO₄ at pH 7.4, and then collected the cells with trypsin into 1 mL of the PBS medium. The 80% confluent cells (approximately 20×10⁶ cells) were suspended in 3 mL of cold isolation buffer that consisted of 0.32 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.5). The cytosolic fractions were obtained as described in a study by Zelewski et al.4 with some modifications. In brief, the cells were homogenized in isolation buffer at 4°C with a Miccra homogenizer and centrifuged at 25000 g for 30 min to obtain supernatant that contained the cytoplasmic enzymes. Partial purification was performed on the supernatant according to the protocol used for clinical tumor samples.

Enzyme assay and kinetic parameters

We measured ME activity in the presence of malate with NADP as the substrate and MgCl₂ as the cofactor. The lowest concentration of each substrate that showed the maximum velocity, the constant rate of product formation, and the linear regressions of the activities for serial dilutions of the enzyme was considered to be the optimum substrate concentration.

The reactions were initiated by the addition of 25 μL of the purified enzyme to 200 μL of the total reaction volume by using 20 mM Tris-HCl buffer (pH 7.5) in the microplate wells. The activity was monitored at 340 nm to assess conversion of NADP to NADPH by means of a Bio Tek Powerwave X2 microplate reader; Kinetic mode, Reading interval = 39 s (BioTek, USA) and Gen5 software version 2.0 (USA). The enzyme activity was expressed as nmoles pyruvate formed/min.

We performed data analysis with Microplate Analysis (MPA) and Kinetics 3.51 software.10,11 The Kinetics 3.51 software fitted the data through nonlinear least squares regression in order to determine the values of the substrate concentration with half-maximal activity (Kₘ; Michaelis-Menten constant) and maximum velocity (Vₘₐₓ).

The Km (malate) was determined at 1 mM NADP with malate concentrations that ranged from 4 to 60 mM. The Km (NADP) was determined at 50 mM malate with NADP concentrations that ranged from 0.0025 to 0.05 mM in both tissues. Of note, 1 mM MgCl₂ cofactor existed in both reaction mixtures and was used for Km determination of malate and NADP.

We calculated both Km and Vₘₐₓ from the mean of three separate series of determinations. The total protein content was measured by the Bradford method with bovine serum albumin as the standard.

Lactate dehydrogenase activity was measured by the addition of 1.5 mM pyruvate to 200 μL total reaction volume in each microplate well. The

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg protein)</th>
<th>Fold purification</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>8.5</td>
<td>0.027</td>
<td>0.0031</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>1.9</td>
<td>0.0155</td>
<td>0.0081</td>
<td>2.61</td>
<td>55</td>
</tr>
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wells contained 0.25 mM NADH, 10 μL of the partially purified samples, and the assay buffer.

Malate dehydrogenase activity was assayed similar to LDH except for the addition of 1 mM oxaloacetate instead of pyruvate to assess MDH activity. The activity of the 2 enzymes was monitored at 340 nm to check the conversion of NADH to NAD.

In order to assess the amount of any conversion of NAD to NADH, eliminate the possible existence of the endogenous conversion of NAD to NADH in the partially purified samples by adding NADP (0.5-1 mM) to the purified samples and monitoring the change in absorbance at 340 nm.

The inhibition constant (I50) and the amount of inhibitor which reduced the enzyme activity by 50% were calculated for guanidine hydrochloride (GuHCl) and NaCL. In this case, we assayed the tumor and normal partially purified ME under optimal conditions for the malate-decarboxylation reaction and with the addition of various concentrations of the above effectors.

**Statistical analysis**
The data were expressed as mean ± SEM from the independent determinations on separate preparations of the enzyme. The data were analyzed using the student’s t-test. The level of significance for all tests was set at P<0.05.

**Results**

**Optimization of the experimental conditions**
The optimum assay conditions for ME were 50 mM malate and 1 mM NADP in both tumor and normal tissues. Of note, there was no NAD-to-NADH conversion activity in the partially purified samples.

### Table 2. The kinetic parameters of malic enzyme (ME) in tumor and normal breast tissues.

<table>
<thead>
<tr>
<th></th>
<th>Tumor</th>
<th>Normal</th>
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<tbody>
<tr>
<td>(K_m) malate (mM)</td>
<td>6.02 ± 1.6*</td>
<td>12.78 ± 4.73</td>
</tr>
<tr>
<td>(K_m) NADP (mM)</td>
<td>0.022 ± 0.004</td>
<td>0.0175 ± 0.0054</td>
</tr>
<tr>
<td>(V_{max}) malate (mU/g wet weight)</td>
<td>35.21 ± 2.29*</td>
<td>14.61 ± 2.7</td>
</tr>
<tr>
<td>(V_{max}) NADP (mU/g wet weight)</td>
<td>42.68 ± 0.51*</td>
<td>34.11 ± 5.61</td>
</tr>
</tbody>
</table>

The assays were conducted at 25 °C and the data are presented as means ± SEM. \(K_m\): Half maximal activity (Michaelis-Menten constant); \(V_{max}\): Maximum velocity. n = 3 independent determinations on each of the tumor and normal samples. *: Significant difference in each row at P<0.05.
The kinetic characteristics of malic enzyme in breast cancer tissue

We determined that the purification procedure used was efficient. Table 1 summarizes the typical purification experiment. DEAE-G50 Sephadex chromatography showed the presence of one peak activity of ME from both tumor and normal breast samples. The peak activity of ME in DEAE-G50 Sephadex consistently eluted at 0.5-1.3 M KCl from both tumor and normal breast specimens. Both LDH and MDH activities were only seen in the washing elution; there was no activity by LDH and MDH in the salt gradient pond (Figure 1). The elution patterns of ME were not significantly different between tumor and normal breast tissues.

The structural characteristics of ME

In the initial structural studies, we exposed ME to increasing amounts of a denaturant (GuHCl). These experiments indicated that the I50 for GuHCl differed between tumor (0.19 ± -0.03) and normal samples (0.16 ± -0.01; Table 3). Additional structural experiments were conducted using common salt (NaCl) and showed that tumor ME was slightly less susceptible to NaCl inhibition compared to normal ME (Table 3).

<table>
<thead>
<tr>
<th>NaCL I50 (M)</th>
<th>Tumor</th>
<th>Normal</th>
</tr>
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<tr>
<td>1.45 ± 0.25</td>
<td>1.32 ± 0.17</td>
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</table>

GuHCl I50 (M) | 0.19 ± -0.03 | 0.16 ± -0.01 |

Table 3. The effect of sodium chloride, a common denaturant, on tumor and normal breast tissues. I50: Inhibition constant; GuHCl: Guanidine hydrochloride; The assays were conducted at 25°C and the data are presented as means ± SEM. n = 3 independent determinations on each of the tumor and normal samples. *: Significant difference in each row at P<0.05.

The kinetic properties of ME

The maximum activity for cancerous ME (C-ME) for pyruvate formation was 35.21 ± 2.29 mU/g for malate and 42.68 ± 0.51 mU/g for NADP. These values were higher than those for normal tissue ME (N-ME), which showed a maximum activity of 14.61 ± 2.7 mU/g for malate and 34.11 ± 5.61 mU/g for NADP (Table 2). The Km (malate) for N-ME (12.78 ± 4.73 mM) was significantly higher than C-ME (6.02 ± 1.6 mM; P<0.05), but no meaningful difference existed between the Km (NADP) in normal (0.0175 ± 0.0054 mM) and tumor tissues (0.022 ± 0.004 mM) as seen in figures 2 and 3.

Figure 2. Lineweaver-Burk plots of malic enzyme (ME) in partially purified breast cancer tissues (n = 10). The data are presented as mean ± SEM. n = 3 independent determinations on separate enzyme samples.
The tumor micro-environment has distinct features in comparison with normal tissues. Highly irregular blood vessels, hypoxia, acidic pH, high interstitial pressure, and low level of nutrients can cause the tumor micro-environment to be a stressful milieu, in which the cells have to adapt to these conditions in order to grow and proliferate. Enzyme function is one of the most effective approaches to construct new metabolic capacities in order to adapt to the stressful milieu. Cells, by changing enzyme kinetics, can appropriately use the substrate or produce useful products that are of greater benefit in a stressful environment. This phenomenon can occur in the tumor environment and the current research has supported this hypothesis.

The NADP-linked ME has 2 cytosolic and mitochondrial isoforms in human tissues. The partial purification procedure was designed to purify cytosolic ME and eliminate the interference of LDH and MDH in the assay for ME activity. The purification results showed that both could be verified by our purification method. In order to prove that the samples were free of mitochondrial ME, we checked the fractions for NADH oxidase activity as a mitochondrial marker. The results indicated that the fractions did not contain any NADH oxidase activity and could be considered free of the mitochondrial particles (e.g., mitochondrial ME). In addition, the enzyme partial purification did not contain LDH and MDH because both enzymes were eluted in the washing elution and ME was eluted in the salt gradient pond.

The data showed that tumor tissue had a higher $V_{\text{max}}$ for ME compared to normal tissues. Thus, the change in enzyme was equivalent to the change of the enzyme $V_{\text{max}}$ because $V_{\text{max}} = K_{\text{cat}}$. Thus, a higher $V_{\text{max}}$ for ME would indicate a high level

![Lineweaver-Burk plots of malic enzyme (ME) in the partially purified breast normal tissues (n = 10). The data are presented as mean ± SEM. n = 3 independent determinations on separate enzyme samples.](image-url)
of enzyme expression in tumors. This interpretation supported results from previous studies, which demonstrated that ME expression had significant upregulation in a variety of human cancers.4

The lower Km of malate and higher maximum velocity of ME in tumors indicated the high affinity of tumor ME to convert malate to pyruvate with concomitant regeneration of NADPH. The high Vmax and affinity for tumor ME indicated that tumor ME tended to change malate to pyruvate at a higher level compared to normal tissue.

This feature of tumor ME has some advantages for cancer cells. One of the most important traits of cancer cells is high aerobic glycolysis.12 Lactate dehydrogenase produces NAD, which is necessary for the progression of aerobic glycolysis.13 Tumor ME, by producing a high level of pyruvate, supports LDH activity to supply the NAD requirement for a continuous flow of aerobic glycolysis, which is essential for the energy production of cancer cells. In order to confirm this interpretation, we assayed LDH activity in the tumor and normal samples. The tumor samples had higher LDH activity compared to normal tissues (data not shown). Secondly, ME produces NADPH through catalyzing the reaction of malate to pyruvate. NADPH from this reaction can be used in different ways, which are vital for cancer cells. NADPH is a major cellular antioxidant, which maintains glutathione in a reductive state to prevent oxidative damage. It is also a necessary cofactor in the reductive biosynthesis of fatty acids.14,15

Given the uncontrolled proliferation rate of cancer cells and the high demand for fatty acids to sustain proliferation, tumor ME via higher production of NADPH can supply the required NADPH for fatty acid synthesis. Abundant production of NADPH could lead to the viability of cells through maintaining caspase 2 in inhibitory state. Thus, it may have an anti-apoptotic effect, which is useful for cancer cells.16 Two additional NADPH-forming metabolic pathways are present in mammalian cells (except ME) – the pentose phosphate pathway (PPP) and isocitrate dehydrogenase (IDH).17 It has been reported that the amount of NADPH generated by ME was trivial compared to the PPP yield and isocitrate dehydrogenase.18-20 It is unlikely that cytosolic ME contributes significantly to the NADPH pool in the cytoplasm of the tumor cells.

Pyruvate is most likely the relevant product of the ME reaction in tumor cells. However, the extra role of NADPH in cancer cells, apart from metabolism, should be considered. NADPH may help cancer cells against some antitumor drugs that have the potential to induce intracellular reactive oxygen species (ROS), which leads to the eventual death of the cancer cells.21,22 Hence, a system that scavenges ROS is vital for survival of cancer cells treated with antitumor drugs. NADPH has a key role in the antioxidant system as a cofactor; thus, ME by providing more NADPH, can have a significant impact on drug-resistant cancer cells.23

Finally, higher maximum activity and the affinity of ME to produce high amounts of pyruvate can cause downstream production of metabolic citrate, which can be used for fatty acid synthesis when converted to acetyl-CoA in the cytoplasm. It can also act in the nucleus to promote core histone acetylation.24

One of the metabolic adaptations of cancer cells is the enhanced consumption of glutamine by reductive carboxylation to sustain the anabolic processes of glutaminolysis.15 The exact molecular mechanism is not entirely clear; however, for the first time, the findings have shown that the higher affinity of ME for malate in tumor tissues could be one of the relevant mechanisms used by cancer cells to expedite glutaminolysis. A higher production of pyruvate by ME in tumor tissues could be used as a precursor for alanine production or NAD generation. The exact fate of malate in the cytoplasm should be studied in depth.

The kinetic differences outlined above indicated that ME from control and tumor tissues might exist in distinct structural states. The initial structural studies concentrated on exposure of ME to increasing amounts of GuHCl (as a denaturant) and were carried out to determine if
ME from tumor and normal tissues was more susceptible to inhibition. The experiment revealed that normal ME was more susceptible than tumor ME. Further structural experiments used common salt, NaCL, which could affect the enzyme structure by disturbing the intermolecular ion-pair interactions. The assay disclosed that tumor ME was slightly less susceptible to NaCL inhibition compared to normal ME. However, it has provided the required evidence that normal and tumor ME exist in distinct structural forms.

The kinetic differences of ME reflect the tumor microenvironment because enzyme function and stability can be strongly influenced by the composition of the intracellular milieu in which the enzyme operates. In order to test this hypothesis, we have evaluated the kinetics of ME in two different breast cancer cell lines (MCF-7 and MDA-MB 231). Of note, the obtained results showed that the Km of malate in both cell lines was nearly 3-fold less than the tumor samples (Table 4). This meaningful difference might be the result of the environment status of the enzymes. The environment of the cell culture would fit the cancer cell’s demands. However, the tumor environment is a stressful niche where cancer cells meet abnormal conditions (e.g., hypoxia, acidic pH, and high interstitial pressure). These two various environments can affect enzyme kinetics as we have observed with ME, which was one of the most significant factors that influenced the enzyme’s kinetics.

In this paper, we have demonstrated that tumor ME has a higher affinity to produce pyruvate from malate. The different kinetics of ME is a mechanism to improve the efficiency of the glutaminolysis and glycolysis pathways in cancer cells. Most studies have suggested that inhibition of glutamine and glutamate dehydrogenase activity\textsuperscript{25,26} is a possible targeted therapy to block glutaminolysis. The current study, for the first time, has shown another possible approach to confront glutaminolysis in cancer cells. If we decrease the affinity, activity, and expression of ME, malate can be presumably converted to oxaloacetic acid at a higher rate and ME cannot support the pyruvate production to sustain glycolysis or acetyl-CoA generation. Further research is needed to confirm this hypothesis. Given the difference of the ME kinetics between tumor and normal samples, the ME capacity as tumor marker should be surveyed in a higher number of breast cancer patients. It is important to note that the substrate preference by tumor ME may be due to the post-translational modification during tumorigenesis. Further investigation is required to detect the post-translational modification of ME in cancer tissues and to identify its effect on the ME structure and the kinetic parameters. Finally, the different Km of malate in tumors and cancer cell lines has not only shown that the results of the cell culture studies differ from tumor tissue investigations, but also showed that the vital requirements of the cell culture systems are similar to the tumor environment.

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**Conflict of Interest**

None declared.

**References**


