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Characterization of cytosolic malic enzyme in human tumor cells

Gerhard Loeber*, Mark B. Dworkin†, Anthony Infante**, Horst Ahorn

Ernst Boehringer Institut, Dr. Boehringergasse 5-11, A-1121 Vienna, Austria

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Abstract

Cytosolic $NADP^+$-dependent malic enzyme (ME) from human tumor cells was characterized in detail and compared to ME from normal human tissues produced in recombinant *E. coli*. Kinetic properties, size as seen in SDS gels, and HPLC elution profiles of tryptic digests of human 'normal cell' ME and $NADP^+$-ME from tumor cells were identical. Thus, $NADP^+$-ME found in tumor cells does not constitute a tumor-specific isoform as suggested by other studies but is identical to the 'housekeeping protein' predominantly expressed in human liver and white adipose tissue.

Key words: Malic enzyme; Glutamine; Tumor cell; Malate; Pyruvate

1. Introduction

Three different isoforms of malic enzyme (ME) are found in mammalian tissues: mitochondrial NAD^+-ME (EC 1.1.1.39), mitochondrial $NADP^+$-ME (EC 1.1.1.40), and cytosolic $NADP^+$-ME (EC 1.1.1.40). Mitochondrial NAD^+-ME is found in rapidly proliferating tissues, particularly tumor cells [1,2]. Mitochondrial $NADP^+$-ME is found in tissues with low division rates such as heart, muscle and brain [3]. Cytosolic $NADP^+$-ME is expressed in liver and adipose tissue [3] and generates NADPH needed for fatty acid biosynthesis. It is under dietary control and can be induced by a carbohydrate-rich diet or thyroid hormones [3,4]. An earlier study [5] suggested that absence or deficiency of $NADP^+$-ME is a marker for malignancy, at least in lymphoid cells. However, significant activities of a cytosolic $NADP^+$-ME with immunological cross-reactivity to cytosolic $NADP^+$-ME from pigeon liver have recently been described in a human breast carcinoma cell line [6,7]. These papers also reported differences in the pI and in the electrophoretic mobility of cytosolic ME from tumor and normal tissues, suggesting the presence of a tumor-specific isoform. We have found cytosolic $NADP^+$-ME in several human tumors cell lines. To determine whether this $NADP^+$-ME differs from the ME in normal tissue, we have cloned this isoform from a human fat cell cDNA library and expressed it in *E. coli*. The nucleotide sequence of the human ME cDNA and its derived amino acid sequence is similar to the $NADP^+$-ME from mouse and rat liver [8,9]. The recombinant protein had identical kinetic properties when compared to cytosolic $NADP^+$-MEs isolated from human liver and different tumor cells. Reverse-phase HPLC of tryptic digests of purified $NADP^+$-ME from human tumor cells and recombinant human ME shows the same elution pattern of the tryptic fragments. This demonstrates that there is no difference between the isoform of cytosolic $NADP^+$-ME expressed in normal and in tumor tissues.

2. Materials and methods

2.1. ME analysis in cell and tissue lysates

A 549 cells (ATCC: CCL185), Colo 205 cells (CCL 222), MCF 7 cells (HTB 22) and RPMI 1788 cells (CCL 156) were grown in DMEM+10% FBS. 2×10^7 cells were washed and collected by centrifugation at $500 \times g$. The pellet was resuspended in 5 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 20 mM KCl, 0.4 mM DTT, 0.5 mM PMSF, 0.05% NP40, 25% glycerol), sonicated and clarified by spinning at $20{,}000 \times g$. Human heart mitochondria were prepared as described in [10]. 5 mg protein were loaded on a Pharmacia 5/5 MonoQ FPLC column and eluted with a 20–180 mM KCl gradient. ME activity was assayed as described in [11]. K_m assays were performed fluorometrically at 40 different malate concentrations between 0 and 0.5 mM.

2.2. Purification of human cytosolic ME from Colo 205 cells

5×10^8 cells were lysed in 50 ml lysis buffer and purified using ion-exchange chromatography (TMAE fractogel; Merck 16881), $NADP^+$-affinity chromatography (Pharmacia 27–5444) and gel-filtration (Superose 6; Pharmacia) in 25 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 0.5 mM EDTA. This procedure yielded a homogeneously pure protein as seen in SDS-polyacrylamide gels.

2.3. Cloning, sequencing and expression of human cytosolic ME

A human fat cell cDNA library (Clontech 1108b) was screened with a cDNA clone of duck ME [12] using radioactive plaque hybridization at reduced stringency [13]. Inserts of positive phages were re-cloned into Bluescript KS(+) using standard procedures [13]. The DNA sequence was determined from the double-stranded plasmid on a ABI373A sequencer with 25 custom made ME-specific primers and fluorescence-

*Corresponding author. Fax: (43) (1) 8010 5683.

**On sabbatical leave from the Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06457, USA.

†Mark B. Dworkin died on 30 April 1992.

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labelled dideoxynucleotides overlapping and for both strands. To express the open reading frame of human cytosolic ME in bacteria, an *Xho*I site was fused to the ATG start codon and a *Hin*dIII site to the stop codon using PCR amplification. This expression cassette was cloned into the expression vector pRH281 as described previously for NAD^+-ME [11]. Recombinant protein was purified as described for ME from Colo 205 cells (above).

2.4. Tryptic digest and HPLC characterization of ME.

40 μg ME from Colo 205 cells or ME from recombinant *E. coli* were digested overnight in 10 μg/ml trypsin (Boehringer-Mannheim; sequencing grade). The resulting peptides were separated with reverse-phase HPLC using a Merck Supersphere column (125-4, 100Å, 4 μM). Peptides were eluted with 70 ml of a linear gradient of 0.1% trifluoroacetic acid in water (A) and acetonitrile (B). The elution profile was monitored at 214 nm.The tryptic peptides were collected, dried and analyzed on a ABI 477A pulsed liquid-phase sequencer.

3. Results

3.1. Characterization of the chromatographic behavior of malic enzyme isoforms

We have found that FPLC ion-exchange chromatography on a MonoQ column allows rapid and reproducible quantification of the different isoforms of malic enzyme (ME). Assays from a crude lysate cannot distinguish between cytosolic and mitochondrial $NADP^+$-ME; other enzymatic activities, such as lactate dehydrogenase (LDH), interfere with the assay for NAD^+-ME. Fig. 1 shows elution profiles of the NAD^+- and $NADP^+$-ME of a human heart lysate, which expresses all three isoforms of ME [3,14]. After loading, the Mono Q columns were eluted with a 20–180 mM KCl gradient. $NADP^+$-ME activity eluted at KCl concentrations of 50 and 130 mM, and NAD^+-ME eluted at 110 mM KCl. Enzymes interfering with the NAD^+-ME assay (LDH isoforms) elute at 30, 80, 90 mM KCl, respectively (data not shown). No ME activity ($NADP^+$ or NAD^+-linked) was in the flowthrough or a 1 M KCl wash of the column of all lysates tested. To characterize the subcellular locations of the ME activities, mitochondria were prepared from human heart muscle and the mitochondrial extract was separated under the same conditions. Fig. 1 shows the presence of $NADP^+$-ME at 50 mM KCl and NAD^+-ME at 110 mM KCl. No $NADP^+$-ME eluted at 130 mM KCl.

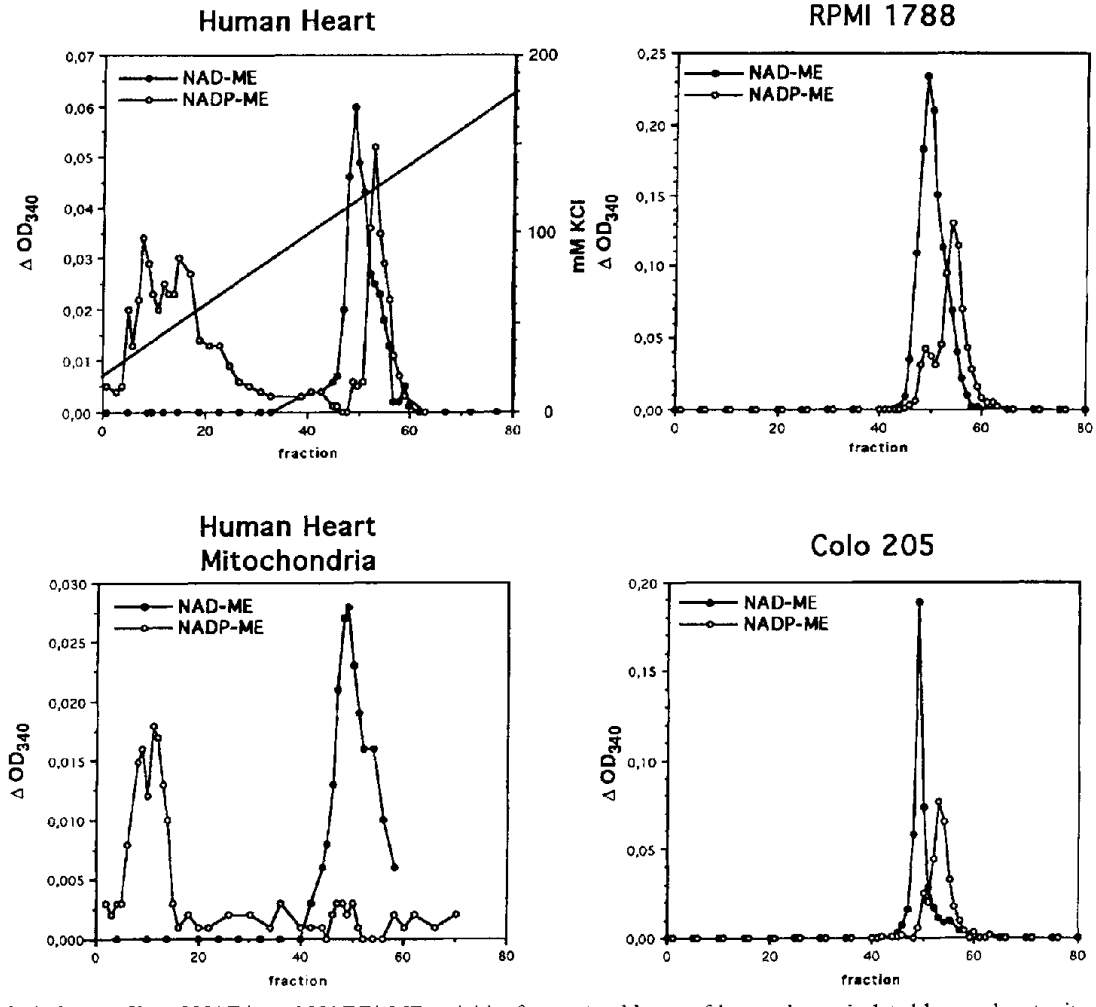

Fig. 1. FPLC elution profiles of NAD^+- and $NADP^+$-ME activities from a total lysate of human heart, isolated human heart mitochondria, Colo 205 and RPMI1788 cells, as indicated. After loading on a MonoQ column, samples were eluted with a 40 ml 20–180 mM KCl gradient with a flow rate of 0.5 ml/min.

Therefore, the enzyme eluting at 130 mM KCl was identified as cytosolic $NADP^+$-ME, the enzyme eluting at 50 mM KCl as mitochondrial $NADP^+$-ME, and the enzyme eluting at 110 mM KCl as mitochondrial NAD^+-ME.

3.2. *Two isoforms of ME are present in tumor cell lines*

Colo 205, A 549 and RPMI1788 cell extracts were separated on MonoQ columns (Fig. 1, Table 1). In all tumor cell lines, activities of NAD^+-ME were found at 110 mM KCl and activities of $NADP^+$-ME were found at 130 mM KCl. No $NADP^+$-ME activity eluted at 50 mM KCl. To verify the absence of mitochondrial $NADP^+$-ME, a separation into cytosolic and mitochondrial fractions was performed and the relative distribution of the respective activities was measured. In Colo 205, A 549 and RPMI1788 cells all NAD^+-ME activity was in the mitochondrial fraction. As expected from the FPLC separations, all tumor cells lacked mitochondrial $NADP^+$-ME activity, whereas significant $NADP^+$-ME activity was present in the cytosolic fraction. Table 1 gives the specific activities (mU ME/mg protein) in tumor cell extracts; they contain considerable amounts of NAD^+- as well as $NADP^+$-ME, with the colon carcinoma and the lymphoid cell line containing about twice as much NAD^+-ME as $NADP^+$-ME.

3.3. *Isolation of a cDNA of cytosolic $NADP^+$-ME from human fat cells and expression in* E. coli

To characterize the properties of the cytosolic ME found in liver and adipose tissue and to compare its properties to those of $NADP^+$-ME from tumor cells, we have isolated and sequenced its cDNA from a human fat cell cDNA library. Our approach to cloning was a plaque hybridization under reduced stringency using a duck liver ME cDNA [12] as a probe. Of a total of 10^6 phage plaques, 20 were positive after plaque purification. One of them coded for a full-length clone of 2,058 bp ending just 1 bp ahead of the ATG start codon. The sequence for the cDNA and the deduced amino acid sequence is shown in Fig. 2. It has an open reading frame of 1,719 bp coding for a protein of 572 amino acids with a molecular weight of 64.1 kDa. The 3′ untranslated region is 339 bp long; a consensus signal for polyadenylation (AAATAA) is located 10 bp upstream of the poly(A)-tail. The encoded protein is similar to cytosolic $NADP^+$-ME cloned from mouse [8] or rat liver [9], showing an identity of 89%. Most of the amino acid changes are conservative exchanges. The cytosolic duck ME [12] is more distantly related to the human isoform; the identity is 77%. Most of the differences between the duck and the human and rat ME are clustered in the N- and C-terminal regions of the protein, with the central portion showing the highest degree of conservation. Compared to human mitochondrial NAD^+-ME [11], significant differences are found. There is only 54% identity between the two human MEs which is close to the 50% homology to $NADP^+$-ME from maize chloroplasts [15]. In both cases, the differences are scattered throughout the protein.

Table 1
Activities of NAD^+- and $NADP^+$-dependent malic enzymes in human tumor cells

Source	$NADP^+$-ME	NAD^+-ME
Colo 205	6.2 ± 0.6	10.9 ± 1.7
A 549	8.0 ± 2.3	6.5 ± 1.9
RPMI 1788	9.3 ± 2.5	20.7 ± 6.3
hu. heart	4.8 ± 0.9	4.1 ± 0.2

Activities of mitochondrial NAD^+- and cytosolic $NADP^+$-ME in tumor cells and normal heart tissue are given in mU/mg of total protein in the extract based on three independently prepared lysates.

To express the coding region of human cytosolic $NADP^+$-ME in *E. coli*, a bacterial ribosome binding site was fused to the 5′ end using PCR amplification. This construct was cloned into the expression vector pRH281T [11] and transfected into *E. coli*. Induction with indoleacrylic acid resulted in high level expression of the recombinant ME. After purification, 1 mg of fully active human $NADP^+$-ME was obtained per liter bacterial broth.

3.4. *Cytosolic tumor cell ME is identical to the 'normal' liver and fat cell ME*

In order to ascertain whether tumor cells express a cytosolic ME which differs from that in normal cells, we compared the recombinant human $NADP^+$-ME to the cytosolic ME from tumor cells. $NADP^+$-ME from Colo 205 cells was purified to near homogeneity. After purification, the protein showed a single band corresponding to a size of 62 kDa in a silver-stained SDS-polyacrylamide gel (Fig. 3). Recombinant human $NADP^+$-ME was purified to homogeneity from *E. coli* using essentially the same procedure as for Colo 205 cells. The purified human recombinant protein had the same mobility in an SDS-polyacrylamide gel as the protein from human tumor cells (Fig. 3). The specific activity for both proteins is 40 mU/μg protein; this is close to the specific activity of 56 mU/μg protein reported for cytosolic $NADP^+$-ME from human liver [16], but significantly higher than the specific activity of 5 mU/μg reported for cytosolic $NADP^+$-ME from a human breast cancer cell line [6]. Since ME is rather unstable in dilute form, differences in the purification protocol may account for these discrepancies. The pH optimum of the recombinant and the colon carcinoma cell ME is about 7.2, and both proteins exhibit about 50% of their optimal activity at pH 6.6. The K_m for malate is 316 μM for colon carcinoma ME, which is comparable to the K_m of 271 μM for recombinant human 'normal cell' ME.

To compare these values with the cytosolic MEs from other tumor cells, we partially purified cytosolic $NADP^+$-ME from MCF 7, A 549, RPMI 1788 cells and from normal human liver and assayed their K_m for malate (Table 2). The K_m was in the same range

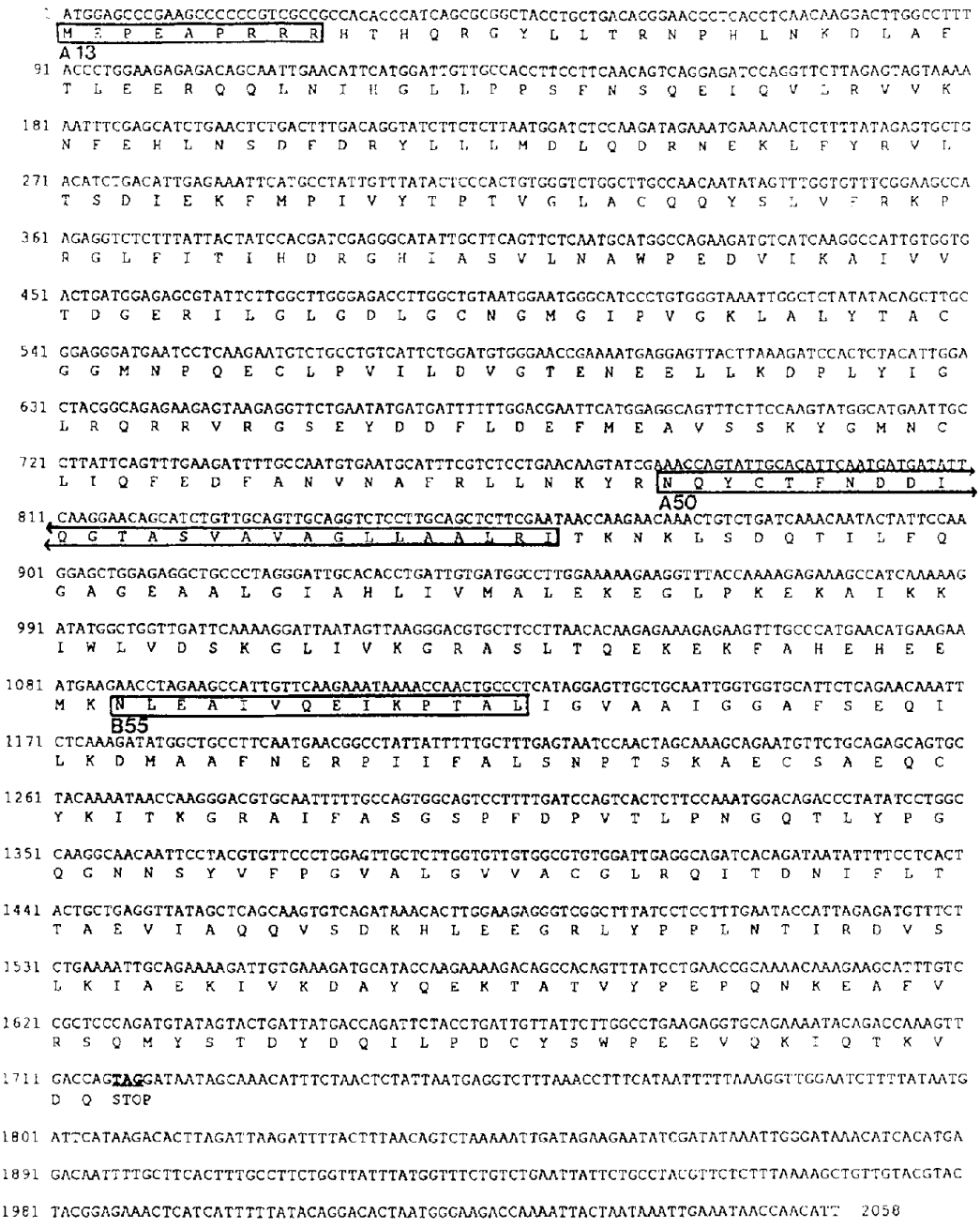

Fig. 2. cDNA and deduced amino acid sequence of the human cytosolic NADP⁺ME. The deduced amino acid sequence is shown below the corresponding nucleotide sequence in the single letter code. Amino acids in boxes correspond to peptide sequences of tryptic peptides of ME found to be different when comparing HPLC elution patterns of human NADP⁺-ME from Colo 205 cells and recombinant *E. coli*. The DNA sequence has been submitted to the Genbank/EMBL Data Bank with the accession number X77244.

(300 ± 50 μM) for all the enzymes derived from the different tumor cell lines and normal tissues, suggesting that the same isoform is expressed. It is also close to the K_m of 216 ± 155 μM for malate reported for the ME isolated from a human breast cancer cell line [6]. As a final step towards the characterization of the cytosolic 'tumor cell' ME we made a tryptic digest of the pure ME from Colo 205 cells and of the recombinant 'normal cell' ME. The digests were loaded onto a reverse-phase HPLC column and eluted under identical conditions. The elution profiles ($OD_{214\,nm}$) are essentially identical; only three significant differences were found (labelled with arrows in Fig. 4). To characterize these differences, we have analyzed the amino acid sequences of the corresponding oligopeptides. Peaks 13 and 50 from the human recombinant ME and peak 55 from the Colo 205 ME were the result of incomplete tryptic digestion, since they contained internal lysines or arginines. The sequences of the oligopeptides of all three peaks were present in the amino acid sequence of the normal cell NADP⁺-ME (boxed in Fig. 2).

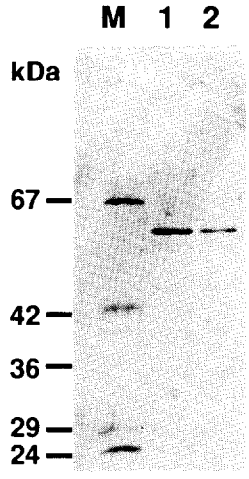

Fig. 3. SDS-polyacrylamide gel electrophoresis of recombinant human cytosolic NADP$^+$-ME produced in *E. coli* (lane 1) and cytosolic NADP$^+$-ME from a human colon carcinoma cells (Colo 205; lane 2). M, molecular weight markers. After separation, the gel was stained using a Bio-Rad silver staining kit.

These data show that cytosolic ME expressed in Colo 205 cells is identical to the NADP$^+$-ME expressed in normal liver and fat cells. Further, it is unlikely that there are any significant posttranslational modifications of the protein in human tumor cells, since the elution pattern of the tryptic digest from reverse phase HPLC should have revealed even minor differences.

4. Discussion

In this study, we have characterized cytosolic NADP$^+$-ME from different human tumor cell lines and compared it to recombinant human cytosolic NADP$^+$-ME expressed in normal tissue. Taken together, the findings are:

(a) There is a substantial amount of cytosolic NADP$^+$-ME in a variety of human tumor cell lines derived from different organs, including colon, breast and lung and in a lymphoid cell line. An earlier study [5] suggested that deficiency of cytosolic ME is a possible marker for malignancy in lymphoid cells. This conclusion cannot be extended to tumor cells derived from tissues of epithelial origin, since all carcinoma cell lines used in this study have considerable NADP$^+$-ME activities. The lymphoid cell line (RPMI1788) used in this report also has significant activities of cytosolic NADP$^+$-ME. However, this finding does not necessarily contradict the report of Povey et al. [5] since RPMI1788 cells are not derived from a malignant leukaemia, but were established from peripheral lymphocytes of a healthy donor. An extended study investigating the status of the different ME isoforms in a large number of human cell lines is currently being done.

(b) We did not find any mitochondrial NADP$^+$-ME in the tumor cell lines tested. This finding differs from a report of an increase in mitochondrial NADP$^+$-ME of preneoplastic and tumor-derived rat tracheal cells when compared to corresponding normal primary cells [17]. However, it is possible that this reported increase in NADP$^+$-ME activity reflected mitochondrial NAD$^+$-ME since, in contrast to the human NAD$^+$-ME which is hardly active with NADP$^+$ [11], rat NAD$^+$-ME has significant activity when assayed with NADP$^+$ [1].

(c) The cytosolic NADP$^+$-ME expressed in the tumor cells used in this study is the same as the ME expressed in normal liver and adipose tissue. Post-translational modifications specific for tumor cells affecting the activity of the protein are unlikely since the recombinant ME has the same size, as seen in SDS-polyacrylamide gels (Fig. 3), the same K_m for malate (Table 2), and the same specific activity (40 mU/μg). Furthermore, reverse-phase HPLC elution is very sensitive and could be expected to have picked up even minor differences if any existed. The differences between cytosolic tumor cell ME and pigeon liver ME described by Chang et al. [6] are most likely due to species-specific differences between the pigeon and the human enzyme; duck liver ME, which is probably very similar to pigeon liver ME, is only 77% identical to human liver ME, and large stretches at the N- and the C-termini of the protein show significant differences.

What is the significance of the expression of cytosolic ME in tumor cells? In normal tissues, this enzyme provides NADPH for fatty acid biosynthesis. However, in a study in progress we found that the NADPH production by ME was small when compared to glucose-6-phosphate dehydrogenase or NADP$^+$-dependent isocitrate dehydrogenase the activity of which is about 50% cytosolic. It is unlikely that cytosolic ME contributes significantly to the NADPH pool in the cytoplasm of the tumor cells. It is more likely that pyruvate is the relevant product of the ME reaction in tumor cells. The significance of mitochondrial NAD$^+$-ME is thought to be related to the use of glutamine as a respiratory fuel, in a pathway from glutamine to lactate that has been named glutaminolysis [18]. This pathway not only provides energy by using a truncated Krebs cycle from α-ketoglutarate to malate, it also produces intermediates for ana-

Table 2
K_m values of human cytosolic NADP$^+$-ME from different tumor cells and normal tissues

A549[a]	313 ± 22 μM
Colo205[b]	316 ± 58 μM
MCF 7[a]	357 ± 82 μM
RPMI 1788[a]	338 ± 93 μM
recomb. human[b]	271 ± 60 μM
human liver[a]	331 ± 73 μM

K_m assays were performed with 3 different and independent preparations of malic enzyme; data were plotted in a Hanes-Woolf plot. Standard deviations are included. [a]Partially purified protein after fractionation on a MonoQ ion-exchange column. [b]Pure protein.

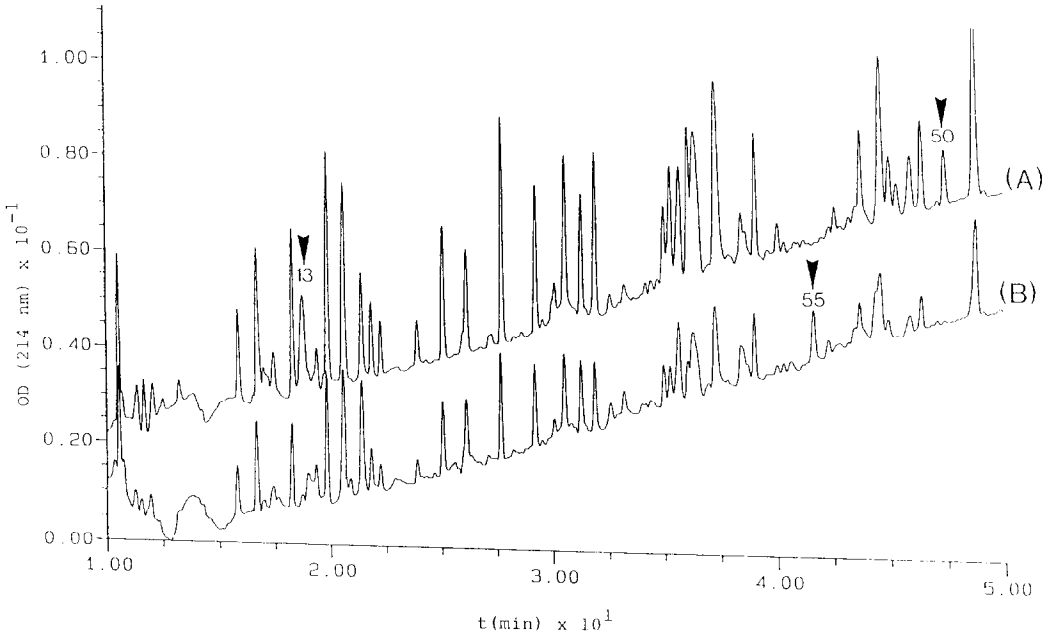

Fig. 4. Reverse-phase HPLC chromatography of tryptic fragments of recombinant human cytosolic NADP$^+$-ME (track A) and cytosolic NADP$^+$-ME from human Colo 205 cells (track B). Significant differences in the elution profile are indicated by arrows. Peptides corresponding to these peaks were collected for sequence analysis.

bolic purposes in tumor cells, e.g. aspartate and nitrogen groups for DNA biosynthesis. Malate generated from glutamine in the mitochondria has three possible options: conversion to oxaloacetate by malate dehydrogenase, decarboxylation to pyruvate in the mitochondria by NAD$^+$-ME, or transport into the cytoplasm. Here, malate can be decarboxylated by cytosolic ME with subsequent conversion to lactate by lactate dehydrogenase. NAD$^+$ produced by this reaction can be used by glyceraldehyde-3-phosphate dehydrogenase, thus compensating for the expression of a partially inactive pyruvate kinase in tumor cells [21]. A significant proportion of glutamine taken up by tumor cells and lymphocytes ends up in lactate, which is secreted [18–20]. Therefore, a possible function of the cytosolic NADP$^+$-ME in tumor cells is the conversion of excess mitochondrial malate, which can cross the mitochondrial membrane readily, to pyruvate, which is subsequently converted to lactate and extruded from the tumor cell.

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