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Properties of Malic Enzyme from the Aerobic Methanotroph *Methylosinus trichosporium*

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Abstract—Recombinant malic enzyme from the aerobic methanotroph *Methylosinus trichosporium* was obtained by heterologous expression in *Escherichia coli* and purified by affinity metal-chelating chromatography. The homohexameric enzyme of 6×80 kDa catalyzed the reversible reaction of oxidative decarboxylation of malate to pyruvate in the presence of mono- and divalent cations and $NADP^+$ as a cofactor. The k_{cat}/K_m ratio indicated much higher catalytic efficiency of the malate decarboxylation reaction as compared with the pyruvate carboxylation reaction. Analysis of the protein sequence revealed that the *C*-region of the enzyme contains a large domain homologous to phosphoacetyltransferase, but no phosphoacetyltransferase activity was detected either for a full chimeric malic enzyme or for the *C*-end fragment obtained as a separate protein. This *C*-end domain promoted activity of the malic enzyme.

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Malic enzyme (ME) is widely distributed in macro- and microorganisms. ME catalyzes the oxidative decarboxylation of malate to pyruvate and CO_2 with the reduction of NAD^+ or $NADP^+$ [1, 2]:

$$\text{Malate} + \text{NAD(P)}^+ \rightleftarrows \text{Pyruvate} + \text{NAD(P)H}_2 + \text{HCO}_3^+.$$

Malic enzymes are divided into three different categories based on their substrate specificity and cofactor dependency: NAD^+-dependent decarboxylating oxaloacetate ME (EC 1.1.1.38), $NAD(P)^+$-dependent ME non-decarboxylating oxaloacetate (EC 1.1.1.39), and $NADP^+$-dependent ME (EC 1.1.1.40) [1-3]. Some malic enzymes use both pyridine nucleotides, but they have different specificities for cofactors. Although it is believed that malic enzymes catalyze a reversible reaction, they differ in their ability to carboxylate pyruvate – from the complete absence of the reaction to its prevalence over the decarboxylation reaction [4, 5].

Aerobic bacteria using methane as a growth substrate (methanotrophs) have an increasing potential for biotechnology; therefore, methods for genetic modification and correction of the metabolism of promising strains are widely used for its successful use. In turn, this requires knowledge of the main biochemical pathways and enzyme properties. The obligate methanotroph *Methylosinus trichosporium* OB3b is a member of the Alphaproteobacteria class and one of the model organisms for studying methylotrophy as the mode for microbial life and for valuation of metabolic potential of these bacteria. *Methylosinus trichosporium* uses the serine pathway for C1 assimilation in which the C3 compound (serine) is the primary product resulting from the condensation of methylene tetrahydrofolate (methylene-THF) and glycine (Fig. 1). Malate, one of the central metabolites of this pathway, is synthesized via transformation of serine through a series of reactions including transamination with glyoxylate, reduction of hydroxypyruvate into glycerate, glycerate phosphorylation, and carboxylation of phosphoenolpyruvate (PEP) into oxaloacetate [6, 7]. The last of these reactions is performed by highly active PEP carboxylase represented by two isoforms [8], and malate is synthesized from oxaloacetate by NADH-dependent

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ME, malic enzyme; PEP, phosphoenolpyruvate; THF, tetrahydrofolate.

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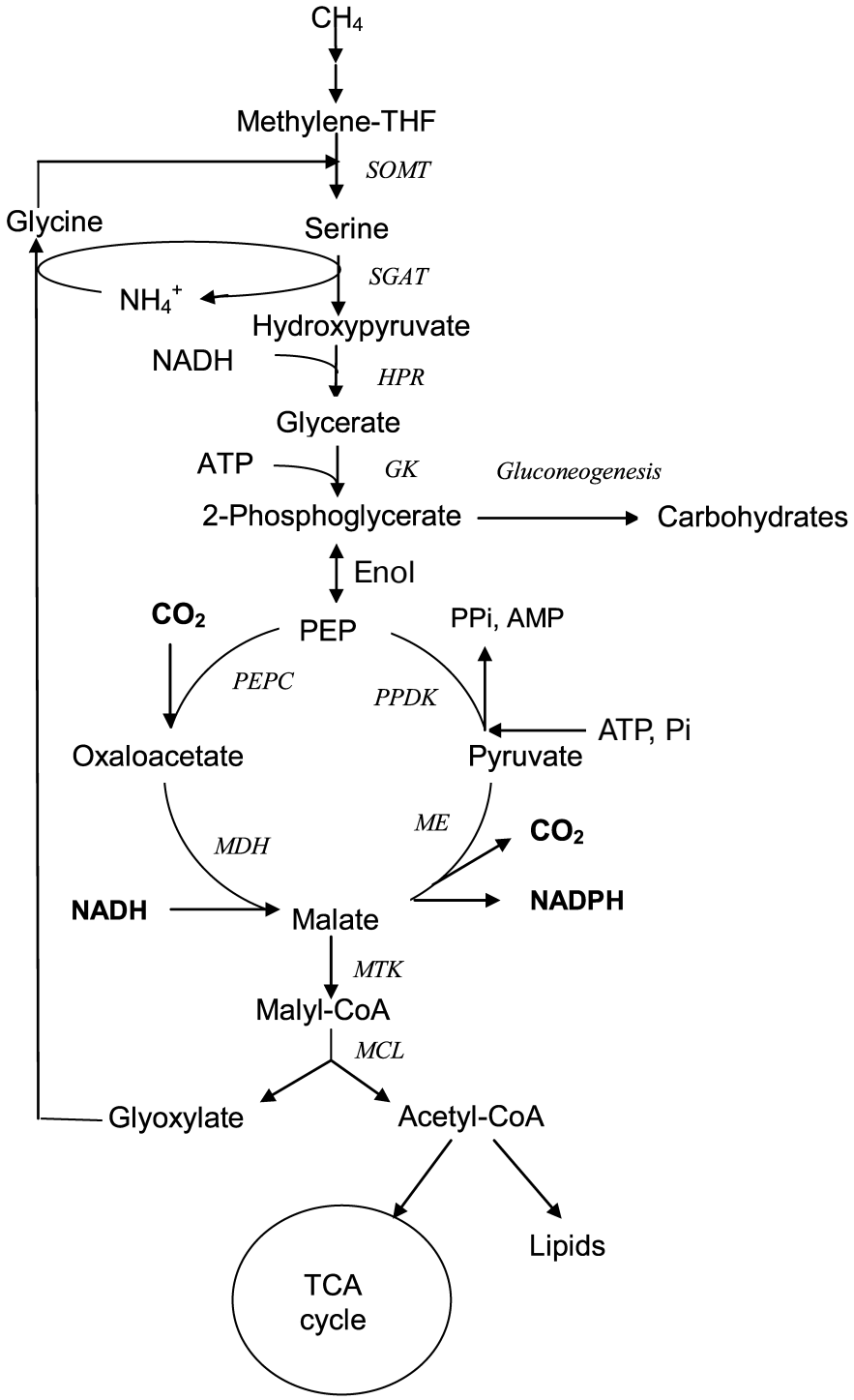

Fig. 1. Putative participation of malic enzyme in central metabolism of *Ms. trichosporium*. SOMT, serine oxymethyltransferase; SGAT, serine glyoxylate aminotransferase; HPR, hydroxypyruvate reductase; GK, glycerate kinase; Enol, enolase; PEPC, PEP carboxylase; MDH, malate dehydrogenase; ME, malic enzyme; PPDK, pyruvate, phosphate dikinase; MTK, ATP-dependent malate thiokinase; MCL, malyl-CoA-lyase; methylene-THF, methylenetetrahydrofolate.

malate dehydrogenase [7]. Further transformation of malate in the serine pathway is associated with the formation of malyl-CoA and the decomposition of this compound into acetyl-CoA and glyoxylate, which is a precursor of glycine, a one-carbon compound acceptor. Thus, the decarboxylating activity of malic enzyme leads to a loss of the C–C bond formed during C1 assimilation. This work aimed at studying the catalytic properties of the

recombinant malic enzyme in order to understand the regulation of this metabolic region in the aerobic methanotroph *Ms. trichosporium*.

MATERIALS AND METHODS

Bacteria and growth conditions. *Methylosinus trichosporium* OB3b (VKM B-2117, ATCC 35070) was grown at 28°C under a methane–air atmosphere (1 : 1 v/v) in nitrate mineral medium "P" [9] containing (g/liter): KNO_3 (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), $CaCl_2$ (0.02), $Na_2HPO_4 \cdot 5H_2O$ (1.5), KH_2PO_4 (0.7), Trilon B (Na_2EDTA) (0.005), $FeSO_4 \cdot 7H_2O$ (0.002), $ZnSO_4 \cdot 7H_2O$ (0.0001), $MnCl_2 \cdot 4H_2O$ (0.00003), $CoCl_2 \cdot 6H_2O$ (0.0002), $CuSO_4 \cdot 5H_2O$ (0.0001), $NiCl_2 \cdot 6H_2O$ (0.00002), $Na_2MoO_4 \cdot 2H_2O$ (0.00003) in a thermostated shaker (200 rpm; New Brunswick Scientific, USA). *Escherichia coli* BL21(DE3) (Novagen, Germany) was grown at 37°C in selective Luria–Bertani agar or broth [10]. For growth of plasmid-bearing cells of *E. coli*, ampicillin was added at concentration of 100 μg/ml.

DNA manipulations. Plasmid isolation, digestion by restriction enzymes, agarose gel electrophoresis, ligation, and transformation of *E. coli* cells were performed according to described methods [10]. Restriction enzymes, T4 DNA-ligase, Pfu and Taq DNA-polymerases, dNTP mixture, and Page Ruler Prestained Protein Ladder for SDS-PAGE were purchased from Thermo Scientific (USA).

Preparing and purification of malic enzyme (ME). Chromosomal DNA from *Ms. trichosporium* cells was prepared as previously described [11]. The *dme* gene (ID 2507408727) encoding putative ME from *Ms. trichosporium* (IMG https://img.jgi.doe.gov) was amplified by PCR using primers N-dme-Nde (5′-TCCATATGGCGGAGAAGCCGCGCATGGACC) and C-dme-Hind (5′-ATAAGCTTCCCCCCCGACGCCGAAGGCCGCCAGC) containing recognition sites for endonucleases NdeI and HindIII, respectively. For expression of *dme*, vector pET22b:dme was constructed and transferred to *E. coli* BL21(DE3) cells. The synthesis of the enzyme was induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at A_{600} = 0.6-0.7. After growth at 18°C for 15 h, the cells were harvested by centrifugation (30 min at 8°C and 5000*g*) and stored at –20°C. The His_6-tagged protein was purified by affinity chromatography on a Ni^{2+}-nitrilotriacetic acid (Ni-NTA) agarose column as described earlier [12]. The purified enzyme was stored in 40% glycerol at –20°C.

For cloning of *N*-terminal sequence of the enzyme-encoding gene (*mae* fragment), the forward primer N-dme-Nde (see above) and the reverse primer 5′-TAAGCTTATCCAGCGCGGCGACCGGATCGCGCC were used. For cloning of the *C*-terminal region of the gene (*patr* fragment) the primers PaTR74-Nde-F (5′-TACATATGCATACGATCTACGATCGCGTGCGGC) and C-dme-Hind were used. The cloning and expression of *mae* and *patr* fragments and purification of the protein were carried out by methods described above.

Determination of molecular masses. The quaternary form of the enzymes were analyzed by non-denaturing gel electrophoresis by using pore-limiting gradient of polyacrylamide (4-30%) [13] with protein marker set (Sigma-Aldrich, Germany): thyroglobulin (660 kDa, dimer), ferritin (440 kDa, 24 subunits), catalase (232 kDa, tetramer), lactate dehydrogenase (140 kDa, tetramer), bovine serum albumin (67 kDa, monomer).

Assay of malic enzyme activity. Activity of the malic enzyme in the direction of malate decarboxylation was determined by measuring $NADP^+$ reduction velocity at 30°C in 1 ml of the reaction mixture containing 50 mM potassium phosphate buffer, pH 7.5, 2.5 mM $MgCl_2$, 0.3 mM $NADP^+$, and ~1 μg of the enzyme. The reaction was initiated by addition of 10 mM malic acid disodium salt. In the direction of pyruvate carboxylation, the enzyme activity was tested by measuring NADPH oxidation in 1 ml reaction mixture containing 50 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 0.25 mM HADPH, 50 mM $KHCO_3$, 25 mM sodium pyruvate, and ~9 μg of the protein.

The ability of ME to catalyze oxaloacetate decarboxylation was tested by two methods. (i) Spectrophotometrically by following the decrease of oxaloacetate absorption at 280 nm [14] in reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5) or MES-NaOH (pH 5.0), 2.5 mM $MgCl_2$, 1-10 mM oxaloacetate, and 50 μg ME in the presence or absence of 0.1 mM $NADP^+$ or NADPH. (ii) By testing the formation of pyruvate in this reaction by HPLC on a Reprosil-Pur c18-AQ column (5 μm, 250 × 10 mm) (Dr. Maisch, Germany) using 1 mM H_2SO_4 and 8 mM Na_2SO_4 as the mobile phase at 25°C and flow rate of 1 ml/min.

The phosphotransacetylase activities of ME and its *C*-terminal fragment were tested by registering the formation of 5-thio(2-nitrobenzoic acid) as a result of the interaction between 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with the sulfhydryl groups of CoA-SH formed during the reaction [15]. The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer, pH 7.5, 0.1 mM DTNB, 2.5 mM $MgCl_2$, and 0.2 mM acetyl-CoA. The reaction was started by adding 10-50 μg of the enzyme. The measurements were carried out at 412 nm.

To study pH dependence of the enzyme activity, the following buffers were used (50 mM): glycine-NaOH (pH 9.0-10.5), CHES-NaOH (pH 8.5-10.0), Tris-HCl (pH 7.6-8.9), potassium phosphate (pH 6.0-8.0), and MES-NaOH (pH 5.0-7.0). The dependence of ME activity on mono- and divalent cations was tested by using aqueous solutions of KCl, NH_4Cl, NaCl (at a final concentration of 50 mM) and $MgCl_2$, $MnCl_2$, $CoCl_2$ (1 mM).

Fructose, glucose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate (at concentration 5 mM), pyruvate, phosphoenolpyruvate, hydroxypyruvate, oxaloacetate, glycerate, α-ketoglutarate, isocitrate, citrate, serine, aspartate (1 mM), ATP, ADP, AMP, PP_i (2 mM) were tested as potential inhibitors or activators of the decarboxylation reaction. To test effect of divalent metals on the enzyme activity, the aqueous solutions of $CuCl_2$, $RbCl_2$, $CdCl_2$, $NiCl_2$, $SnCl_2$, $CoCl_2$, $BaCl_2$, $ZnCl_2$, or $CaCl_2$ at a final concentration of 1 mM were added in the standard reaction mixture containing 1 mM $MnCl_2$ (instead of 2.5 mM $MgCl_2$). To determine the thermal stability, aliquots of the concentrated enzyme in Eppendorf (Germany) tubes were incubated at 30, 40, 50, 60, and 70°C from 5 min to 3 h. After heating, an aliquot was diluted 50-fold by the cooled buffer, and residual activity was determined at 30°C. The percentage of residual activity was calculated by comparison with the non-incubated enzyme. To search for the optimal temperature for activity, the reaction velocity was measured at 10-70°C. For calculation of K_m, the enzyme activity was measured by varying substrates in the concentration range of 0.156-10 mM (malate), 0.0047-0.25 mM ($NADP^+$), 1.56-25 mM (pyruvate), and 0.0156-0.375 mM (NADPH). K_m and V_{max} values were calculated using SigmaPlot (v.10). Protein concentrations were assayed by a modification of the Lowry method [16]. NADPH oxidation/formation rates were followed at 340 nm on a Shimadzu UV-1700 spectrophotometer (Japan).

RESULTS

Preparation and purification of the malic enzyme. The *dme* gene encoding putative malic enzyme from the *Ms. trichosporium* genome was expressed in *E. coli* BL21(DE3). The recombinant protein was purified from the *E. coli* cell extract by single-stage metal chelating chromatography. SDS-PAGE of the protein revealed a single band corresponding to a molecular mass of ~80 kDa, which corresponded with the theoretically calculated (81.5 kDa) (Fig. 2). According to electrophoresis under non-denaturing conditions, the protein mass is 480 kDa, which indicates its hexameric organization. Earlier, dimeric, tetrameric, and octameric forms have been detected in microbial malic enzymes. $NADP^+$-ME from *E. coli*, as well as $NADP^+$-ME and $NAD(P)^+$-ME from *Sinorhizobium meliloti*, were found to be homooctamers [17, 18], while $NADP^+$-ME from *Bradyrhizobium japonicum* was a dimer or tetramer depending on pH values (pH 8.0 and 7.2, respectively) [19].

Catalytic properties. The malic enzyme catalyzed the decarboxylation of malate to pyruvate using $NADP^+$ as a cofactor. It did not use NAD^+ and did not show activity of oxaloacetate decarboxylation. The enzyme activity was strongly dependent on both mono- and divalent cations:

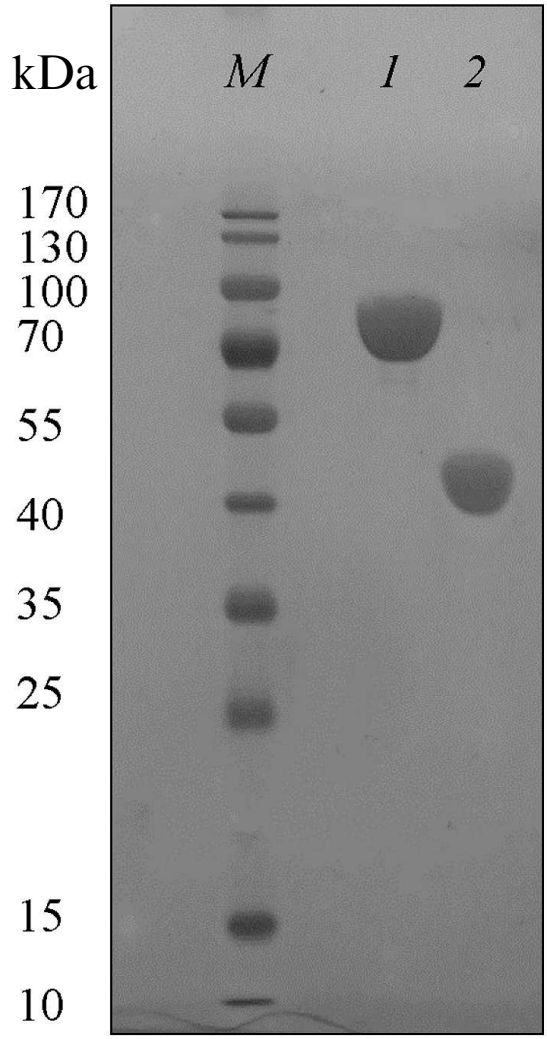

Fig. 2. SDS-PAGE of *Ms. trichosporium* malic enzyme (*1*) and ME1 (*N*-end fragment of *Ms. trichosporium* ME) (*2*). M, markers of molecular masses.

K^+ (or NH_4^+) and Mn^{2+} (or Mg^{2+}) (Table 1). At the same time, in the presence of 50 mM KCl, 50 mM NaCl inhibited its activity by 40%, and $KHCO_3$, but not $NaHCO_3$, served as a CO_2 donor for carboxylation of pyruvate. The enzyme was active in wide ranges of pH (pH 6.0-9.0) and temperature (20-70°C), demonstrating maximum of activity at pH 7.0 and 65°C. ME showed moderate thermal stability: the enzyme activity was not decreased after incubating the protein for 3 h at 30-40°C, but decreased by half after heating for 1 h at 50°C, and 80% activity was lost after 5 min heating at 60°C.

The dependence of the activity on the concentration of substrates obeyed Michaelis–Menten kinetics. At 30°C and optimal pH, apparent K_m values were 2.7 ± 0.3 mM for malate, 64 ± 9 μM for $NADP^+$, 6.0 ± 0.8 mM for pyruvate, and 47 ± 4 μM for NADPH. The enzyme demonstrated 4.7-fold higher activity in the direction of

Table 1. Influence on K^+, NH_4^+, Na^+ (50 mM), and divalent metals (1 mM) on the activity of the recombinant malic enzyme from *Ms. trichosporium*

Cations	Activity, %
Without cations	< 0.01
K^+	8.2 ± 0.4
Na^+	< 0.01
NH_4^+	20 ± 2
Mg^{2+}	3.1 ± 0.3
K^+, Mg^{2+}	100 ± 2.8
Na^+, Mg^2	4.2 ± 0.3
NH_4^+, Mg^{2+}	115.2 ± 6.3
K^+, Mn^{2+}	123.3 ± 8.1
K^+, Co^{2+}	73.8 ± 5.6
K^+, Na^+, Mg^{2+}	59.9 ± 4.2

malate decarboxylation compared with pyruvate carboxylation (Table 2). As followed from the k_{cat}/K_m values, the efficiency of the enzyme in the decarboxylation reaction was an order of magnitude higher than that in the carboxylation reaction. Hydroxypyruvate at concentration of 1 mM inhibited activity of ME by 45% (Table 3). Acetyl-CoA (0.1 mM) exerted the greatest inhibitory effect, in the presence of which the residual activity was 24%. ATP and PP_i at a concentration of 2 mM inhibited the enzyme by 50%, but the activity was completely recovered if Mg^{2+} concentration increased to 5 mM. In the presence of Mn^{2+}, Cd^{2+} ions almost completely inhibited the activity (5.9% residual activity), and Sn^{2+} ions reduced activity by 31%. The other metals tested (see "Materials and Methods") did not have a significant effect on the ME activity.

Effect of the *C*-terminal domain on activity of the malic enzyme. In the amino acid sequence of *Ms. trichosporium* ME, the *N*-terminal fragment consisting of 437 a.a. homologous to malic enzymes (ME1) and an extended *C*-terminal sequence of 322 a.a. corresponding to phosphoacetyltransferases (EC 2.3.1.8) were found. A similar domain structure has been previously described for $NADP^+$-dependent malic enzymes from bacteria, such as *E. coli* and *Sinorhizobium meliloti*, as well as from plants [17, 18, 20].

By cloning of the DNA sequences, two separate proteins, ME1 and the putative phosphoacetyltransferase, were obtained and purified. The specific activities of ME1 in decarboxylation and carboxylation directions were 12 and 1.6 U/mg protein, respectively. At the same time, the apparent K_m values for carbon substrates were higher for ME1 as compared to the initial two-domain protein (Table 2). Unlike the full-length protein, acetyl-CoA did not affect the ME1 activity, while the inhibiting effect of hydroxypyruvate was maintained. Neither the complete chimeric protein nor the phosphoacetyltransferase domain transfer acetyl groups and formation of CoA-SH from acetyl-CoA.

Protein ME1 had a molecular weight of 90 kDa, which corresponded to a dimeric structure, whereas the phosphoacetyltransferase fragment was a hexamer with a molecular mass of 210 kDa. Obviously, the *C*-terminal 322 a.a. fragment was responsible for optimal oligomerization of the malic enzyme. A similar assumption has been made for two malic enzymes from *Sinorhizobium meliloti* [17]. Phosphoacetyltransferase activity was not detected for any of the studied chimeric malic enzymes.

DISCUSSION

In this work, we first described the malic enzyme from the obligate methanotroph *Ms. trichosporium*. The enzyme belongs to the group of $NADP^+$-dependent malic enzymes. Both divalent metal ions (Mn^{2+} or Mg^{2+}) and monovalent cations (K^+ or NH_4^+) were necessary for the enzyme activity, whereas Na^+ ions had an inhibitory effect. Activation by NH_4^+ cations has been found to be a characteristic feature of $NADP^+$-dependent malic enzymes (for example, ME from *Clostridium thermocellum*) [21] and of the NAD^+-dependent ones (from *Streptococcus bovis*) [22]. However, K^+ ions had only a small stimulating effect on the NAD^+-ME from *S. bovis*. Evidences on the Na^+ inhibition on the malic enzyme has not been reported in the literature.

Like other $NADP^+$ enzymes, ME from *Ms. trichosporium* catalyzed a reversible reaction, while the activity of pyruvate carboxylation was significantly lower than malate decarboxylation. In addition, the enzyme from *Ms. trichosporium* had a high K_m for pyruvate (~6 mM) relative to its physiological intracellular concentrations. Although the pyruvate content in cells of this methanotroph can reach high values (>1 mM [23]), such concentration still could not support effective CO_2 assimilation. High values of apparent K_m for pyruvate have been found for $NADP^+$-ME from *E. coli* and *Corynebacterium glutamicum* (6.21 and 13.8 mM, respectively) [18, 24]. Interestingly, only the enzyme from the hyperthermophilic archaea *Thermococcus kodakaraensis* had lower K_m to pyruvate than to malate (7.3 and 16.9 mM, respectively) [5], although the catalytic efficiencies of the decarboxylation and carboxylation reactions were almost the same. In addition, similarly to $NADP^+$-ME from *Clostridium thermocellum* [21] and *Thermococcus kodakaraensis* [5], ME from *Ms. trichosporium* could not decarboxylate oxaloacetate.

ME activity from *Ms. trichosporium* was inhibited by intermediates of the carbon assimilation pathway – hydroxypyruvate and acetyl-CoA. Typically, the activities of bacterial malic enzymes were affected by the TCA intermediates. Malate, succinate, and fumarate activated, but acetyl-CoA inhibited the $NAD(P)^+$-dependent enzyme

Table 2. Kinetic parameters of the *Ms. trichosporium* malic enzyme and its *N*-terminal fragment (ME1)

Parameters	ME	ME1
Molecular mass (number of subunits), kDa	480 (80 × 6)	90 (45 × 2)
V_{max}, U/mg protein (malate → pyruvate)	36 ± 2	12.0 ± 0.3
V_{max}, U/mg protein (pyruvate → malate)	8.0 ± 0.4	1.60 ± 0.03
Inhibitors (residual activity)	0.2 mM acetyl-CoA (24%), 1 mM hydroxypyruvate (55%)	1 mM hydroxypyruvate (64%)
	K_m	
Malate, mM	3.0 ± 0.3	4.0 ± 0.3
$NADP^+$, μM	64 ± 9	ND
Pyruvate, mM	6.0 ± 0.8	11 ± 1
NADPH, μM	47 ± 4	ND
$k_{cat\ malate}$, 1/min	18	1
k_{cat}/K_m^{malate}, 1/(mM·min)	7	0.3
$k_{cat\ pyruvate}$, 1/min	4	0.1
$k_{cat}/K_m^{pyruvate}$, 1/(mM·min)	0.6	0.01

Note: ND, not determined.

Table 3. Activity of the *Ms. trichosporium* malic enzyme in the presence of some metabolites

Effector (concentration)	Residual activity, %
Without effector	100
Oxaloacetate (1 mM)	97 ± 3
Isocitrate (1 mM)	98 ± 4
Citrate (1 mM)	84 ± 4
α-Ketoglutarate (1 mM)	103 ± 1
Succinate (1 mM)	97 ± 1
Glutamate (1 mM)	110 ± 1
Phosphoenolpyruvate (1 mM)	98 ± 2
Pyruvate (1 mM)	104 ± 2
Hydroxypyruvate (1 mM)	55 ± 3
Serine (1 mM)	97 ± 3
Aspartate (1 mM)	108 ± 2
Glucose (5 mM)	93 ± 2
Glucose 6-phosphate (5 mM)	89 ± 3
Fructose (5 mM)	93 ± 2
Fructose 6-phosphate (5 mM)	93 ± 1
Fructose 1,6-bisphosphate (5 mM)	80 ± 1
ATP (2 mM)	47 ± 4
ATP (2 mM), $MgCl_2$ (5 mM)	89 ± 3
ADP (2 mM)	96 ± 3
AMP (2 mM)	103 ± 1
PPi (2 mM)	46 ± 2
PPi (2 mM), $MgCl_2$ (5 mM)	89 ± 3
CoA (0.1 mM)	94 ± 2
Acetyl-CoA (0.2 mM)	24 ± 3

from *Sinorhizobium meliloti*, while $NADP^+$-ME did not undergo any regulation [25]. $NADP^+$-ME activity from *E. coli* was inhibited by fumarate, oxaloacetate, and acetyl-CoA, whereas glutamate, aspartate, glucose-6-phosphate, and acetyl phosphate activated this enzyme [18].

Malic enzyme from *Ms. trichosporium* consisted of two fragments. The *N*-terminal polypeptide obtained as independent protein had low activity and was not inhibited in the presence of acetyl-CoA. Since the diminished version of the malic enzyme (in the absence of the *C*-terminal fragment) was a dimer, but not a hexamer, unlike the full-length protein, the role of the *C*-terminal sequence in the optimal enzyme configuration was suggested. Considering the absence of inhibition, the "chimeric" $NADP^+$-dependent malic enzyme from *Sinorhizobium meliloti* by acetyl-CoA, the impact of the additional fragment in interaction of effectors with the enzyme requires further study.

According to the kinetic properties, the malic enzyme from *Ms. trichosporium* catalyzed malate decarboxylation more efficiently than pyruvate carboxylation; therefore, it can be considered as a "lipogenic" enzyme that produces NADPH, which is necessary for the synthesis of fatty acids and steroids. Such a function was proposed for a number of bacterial $NADP^+$-ME [1, 2, 26].

In *Ms. trichosporium*, malate is formed by carboxylation of phosphoenolpyruvate (PEP) by highly active PEP carboxylase [8] followed by reduction of the oxaloacetate by NADH-dependent malate dehydrogenase [7]. The subsequent decarboxylation of malate by the malic enzyme leads to the synthesis of NADPH (Fig. 1). Consequently, of the activity of the three listed enzymes, PEP is transformed into pyruvate, which is accompanied by the consumption of NADH and the formation of NADPH:

$$PEP + NADH + NADP^+ \rightarrow Pyruvate + NAD^+ + NADPH.$$

In the case of the functioning of pyruvate, phosphate dikinase (ID 2507410009), PEP can be regenerated with the consumption of an ATP molecule and formation of PP_i and AMP in a total reaction of the futile cycle:

$$NADH + NADP^+ + ATP + P_i \rightarrow$$

$$\rightarrow NADPH + NAD^+ + AMP + PP_i.$$

The substitution of ATP for PP_i in this cycle correlates with an important metabolic role of PP_i in methanotrophs [27]. *Methylosinus trichosporium* can synthesize NADPH by the $NADP^+$-dependent isocitrate dehydrogenase [8]. However, the function of the malic enzyme as a source of NADPH does not seem to be redundant, since this methanotroph has high requirements for NADPH, which is necessary for the synthesis of fatty acids and the formation of a system of intracytoplasmic membranes, where methane is oxidized by the particulate (membranous) methane monooxygenase.

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

This article does not include research carried out by the authors with the participation of people or animals.

REFERENCES

1. Chang, G. G., and Tong, L. (2003) Structure and function of malic enzymes, a new class of oxidative decarboxylases, *Biochemistry*, **42**, 12721-12733.
2. Sauer, U., and Eikmanns, B. J. (2005) The PEP–pyruvate–oxaloacetate node as the switch point for carbon flux distribution in bacteria, *FEMS Microbiol. Rev.*, **29**, 765-794.
3. Viljoen, M., Subden, R. E., Krizus, A., and Van Vuuren, H. J. (1994) Molecular analysis of the malic enzyme gene (*mae2*) of *Schizosaccharomyces pombe*, *Yeast*, **10**, 613-624.
4. Liguori, M., Tessarolo, D., Abbruzzese, C., and Giacanelli, M. (1995) $NAD^+/NADP^+$-dependent malic enzyme: evidence of a $NADP^+$ preferring activity in human skeletal muscle, *Biochem. Mol. Med.*, **56**, 14-18.
5. Fukuda, W., Ismail, Y. S., Fukui, T., Atomi, H., and Imanaka, T. (2005) Characterization of an archaeal malic enzyme from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1, *Archaea*, **1**, 293-301.
6. Trotsenko, Y. A., and Murrell, J. C. (2008) Metabolic aspects of aerobic obligate methanotrophy, *Adv. Appl. Microbiol.*, **63**, 183-229.
7. Rozova, O. N., Khmelenina, V. N., Bocharova, K. A., Mustakhimov, I. I., and Trotsenko, Y. A. (2015) Role of NAD^+-dependent malate dehydrogenase in the metabolism of *Methylomicrobium alcaliphilum* 20Z and *Methylosinus trichosporium* OB3b, *Microorganisms*, **3**, 47-59.
8. Matsen, J. B., Yang, S., Stein, L. Y., Beck, D., and Kalyuzhnaya, M. G. (2013) Global molecular analyses of methane metabolism in methanotrophic Alphaproteobacterium, *Methylosinus trichosporium* OB3b. Part I. Transcriptomic study, *Front. Microbiol.*, **7**, 1-13.
9. Galchenko, V. F. (2001) *Methanotrophic Bacteria* [in Russian], GEOS, Moscow.
10. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Edn., Cold Spring Harbor Laboratory, New York.
11. Kalyuzhnaya, M., Khmelenina, V. N., Kotelnikova, S., Holmquist, L., Pedersen, K., and Trotsenko, Y. A. (1999) *Methylomonas scandinavica* sp. nov., a new methanotrophic psychrotrophic bacterium isolated from deep igneous rock ground water of Sweden, *Syst. Appl. Microbiol.*, **22**, 565-572.
12. Reshetnikov, A. S., Rozova, O. N., Khmelenina, V. N., Mustakhimov, I. I., Beschastny, A. P., Murrell, J. C., and Trotsenko, Y. A. (2008) Characterization of the pyrophosphate-dependent 6-phosphofructokinase from *Methylococcus capsulatus* Bath, *FEMS Microbiol. Lett.*, **288**, 202-210.
13. Slater, G. G. (1969) Stable pattern formation and determination of molecular size by pore-limit electrophoresis, *Anal. Chem.*, **41**, 1039-1041.
14. Sender, P. D., Martin, M. G., Peiru, S., and Magni, C. (2004) Characterization of an oxaloacetate decarboxylase that belongs to the malic enzyme family, *FEBS Lett.*, **570**, 217-222.
15. Bock, A. K., Glasemacher, J., Schmidt, R., and Schonheit, P. (1999) Purification and characterization of two extremely thermostable enzymes, phosphate acetyltransferase and acetate kinase, from the hyperthermophilic eubacterium *Thermotoga maritima*, *J. Bacteriol.*, **181**, 1861-1867.
16. Shacterle, G. R., and Pollack, R. L. (1973) A simplified method for quantitative assay of small amounts of protein in biological material, *Anal. Biochem.*, **51**, 654-657.
17. Mitsch, M. J., Voegele, R. T., Cowie, A., Osteras, M., and Finan, T. M. (1998) Chimeric structure of the $NAD(P)^+$- and $NADP^+$-dependent malic enzymes of *Rhizobium (Sinorhizobium) meliloti*, *J. Biol. Chem.*, **273**, 9330-9336.
18. Bologna, F. P., Andreo, C. S., and Drincovich, M. F. (2007) *Escherichia coli* malic enzymes: two isoforms with substantial differences in kinetic properties, metabolic regulation, and structure, *J. Bact.*, **189**, 5937-5946.
19. Chen, F., Okabe, Y., Osano, K., and Tajima, S. (1997) Purification and characterization of the NADP-malic enzyme from *Bradyrhizobium japonicum* A1017, *Biosc. Biotech. Biochem.*, **61**, 384-386.
20. Troconi, M. A., Andreo, C. S., and Drincovich, M. F. (2018) Chimeric structure of plant malic enzyme family:

different evolutionary scenarios for NAD- and NADP-dependent isoforms, *Front. Plant Sci.*, **9**, 1-15.

21. Taillefer, M., Rydzak, T., Levin, D. B., Oresnik, I. J., and Sparling, R. (2015) Reassessment of the transhydrogenase/malate shunt pathway in *Clostridium thermocellum* ATCC 27405 through kinetic characterization of malic enzyme and malate dehydrogenase, *Appl. Environ. Microbiol.*, **81**, 2423-2432.
22. Kawai, S., Suzuki, H., Yamamoto, K., Inui, M., Yukawa, H., and Kumagai, H. (1996) Purification and characterization of a malic enzyme from the ruminal bacterium *Streptococcus bovis* ATCC 15352 and cloning and sequencing of its gene, *Appl. Environ. Microbiol.*, **62**, 2692-2700.
23. Yang, S., Matsen, J. B., Konopka, M., Green-Saxena, A., Clubb, J., Sadilek, M., Orphan, V. J., Beck, D., and Kalyuzhnaya, M. G. (2013) Global molecular analyses of methane metabolism in methanotrophic Alphaproteobacterium, *Methylosinus trichosporium* OB3b. Part II. Metabolomics and ^{13}C-labeling study, *Front. Microbiol.*, **4**, 70.
24. Gourdon, P., Baucher, M.-F., Lindley, N. D., and Guyonvarch, A. (2000) Cloning of the malic enzyme gene from *Corynebacterium glutamicum* and role of the enzyme in lactate metabolism, *Appl. Environ. Microbiol.*, **66**, 2981-2987.
25. Driscollt, B. T., and Finan, T. M. (1997) Properties of NAD^+- and $NADP^+$-dependent malic enzymes of *Rhizobium (Sinorhizobium) meliloti* and differential expression of their genes in nitrogen-fixing bacteroids, *Microbiology*, **143**, 489-498.
26. Ratledge, C. (2014) The role of malic enzyme as the provider of NADPH in oleaginous microorganisms: a reappraisal and unsolved problems, *Biotechnol. Lett.*, **36**, 1557-1568.
27. Khmelenina, V. N., Rozova, O. N., Akberdin, I. R., Kalyuzhnaya, M. G., and Trotsenko, Y. A. (2018). Pyrophosphate-dependent enzymes in methanotrophs: new findings and views, in *Methane Biocatalysis: Paving the Way to Sustainability* (Kalyuzhnaya, M. G., and Xing, X. H., eds.) Springer, International Publishing AG, Switzerland, pp. 83-98.