Operation of glyoxylate cycle in halophilic archaea: presence of malate synthase and isocitrate lyase in *Haloferax volcanii*

Juan Antonio Serrano, Mónica Camacho, Maria José Bonete*

División de Bioquímica, Facultad de Ciencias, Universidad de Alicante, Ap. 99, 03080 Alicante, Spain

Received 13 May 1998; revised version received 13 July 1998

Abstract The occurrence of the glyoxylate cycle has not previously been demonstrated in any of the Archaea. In halophilic archaea, only isocitrate lyase activity has been detected. The halophilic archaeon *Haloferax volcanii* was tested for the presence of the other key enzyme of this pathway, malate synthase. High activities of this enzyme were detected when the carbon source was acetate. Both glyoxylate cycle key enzymes, isocitrate lyase and malate synthase, from *Hf. volcanii* were purified and characterized.

© 1998 Federation of European Biochemical Societies.

Key words: Glyoxylate cycle; Malate synthase; Isocitrate lyase; Isocitrate dehydrogenase; Archaeon; *Haloferax volcanii*

1. Introduction

The glyoxylate cycle is a metabolic pathway by which organisms can synthesize carbohydrates from C2 compounds. This cycle has some enzymes in common with the citrate cycle, and there are two exclusive enzymes: isocitrate lyase (EC 4.1.3.1), which catalyzes the cleavage of D-isocitrate to glyoxylate and succinate; and malate synthase (EC 4.1.3.2), which catalyzes the formation of L-malate from glyoxylate and acetyl-CoA. The net function of glyoxylate cycle is the formation of a C4 compound from two acetyl-CoA (C2) molecules in each cycle. There are alternative pathways to the glyoxylate cycle in some bacteria [1,2].

Taking into account the three domains of living organisms (Bacteria, Eukarya and Archaea) [3], the key enzymes of this cycle (isocitrate lyase and malate synthase) have been detected in Bacteria and Eukarya (fungi, plants, some invertebrates [4], and even in some vertebrates [5,6]). In Bacteria and unicellular Eukarya, the glyoxylate cycle operates mainly when the principal or sole carbon source is a C2 compound (acetate, ethanol). In all organisms this pathway can operate to generate carbohydrates from stored lipids. This is very important in plants during germination of seedlings, and recently the importance of the glyoxylate cycle in animals has been pointed out [4] (for review see [7,8]).

Recent works indicate an unsuspected diversity of archaeal organisms [9], which leads us to think of an important role of Archaea in the biosphere. Despite this and the importance of the glyoxylate cycle, there are no reports that confirm its operation in any of the Archaea. Only isocitrate lyase activity has been clearly detected in some genera of halophilic Archaea [10]. That activity was induced when acetate was the main carbon source. Malate synthase activity could not be detected in that study. There is an earlier work [11], from 1969, in which isocitrate lyase and malate synthase activities were detected in the archaeon *Halobacterium salinarum*, but data are poor, values of activity are very feeble, and those results could not be repeated in the cited article [10].

Acetate could have a role in the nutrition of natural communities of halophilic Archaea, as it is produced from glycerol (the main carbon source in nature) in hypersaline lakes by some species of halophiles [12]. *Haloferax volcanii* is a halophilic archaeon able to grow in minimal medium with acetate as the sole carbon source [13]. Isocitrate lyase activity was detected in this organism when it was grown on a medium with acetate as the main carbon source [10]. In view of these previous works we decided to investigate the assimilation of acetate by the archaeon *Hf. volcanii* and prove the presence of malate synthase, an enzyme that has not previously demonstrated in Archaea.

2. Materials and methods

2.1. Culture conditions and cell extracts

Hf. volcanii was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 3757). It was grown aerobically at 37°C, in a chemically defined medium [13], with 0.1% sodium acetate or 0.5% sodium lactate as carbon source.

Cells were harvested by centrifugation (30 min, $9600\times g$); suspended in extraction buffer; and lysed by sonication in a bath of ice. Cell debris was removed by centrifugation (50 min, $105\,000\times g$) and supernatant was used in enzyme assays and purification. The extraction buffer was 50 mM Tris, pH 7.5; 3 M KCl when cell extracts were used only to assay activity.

2.2. Enzyme assays and determination of protein concentration

Isocitrate lyase was assayed spectrophotometrically at A_{324}, according to the rate of formation of glyoxylate phenylhydrazone ($\varepsilon = 1.7\times10^4$ M^{-1} cm^{-1}) in the presence of isocitrate and phenylhydrazine. It was made at 40°C, and as described in [10] (70 mM HEPES buffer, pH 7.0; 1.9 M KCl; 5 mM $MgCl_2$; 4 mM phenylhydrazine; 4 mM D,L-isocitrate). One unit of enzyme activity is defined as the amount of enzyme necessary to produce 1 μmol of glyoxylate phenylhydrazone per minute under assay conditions. The pH was varied using PIPES in the range of 6.1–7.0 and HEPES between 7.0 and 8.0.

Malate synthase activity was measured according to the rate of formation of 5-mercapto-2-nitrobenzoic acid (CoASH+DTNB) in the presence of glyoxylate, acetyl-CoA and DTNB. The increase in absorbance at 412 nm ($\varepsilon = 1.36\times10^4$ M^{-1} cm^{-1}) was measured spectrophotometrically, at 40°C, in a reaction mixture containing: 20 mM Tris buffer, pH 8.0; 2 mM EDTA; 3 M KCl; 5 mM $MgCl_2$; 0.1 mg/ml DTNB; 0.5 mM glyoxylate and 0.2 mM acetyl-CoA. The reaction was started by the addition of acetyl-CoA. One unit of enzyme activity is defined as the amount of enzyme necessary to produce 1 μmol of 5-mercapto-2-nitrobenzoic acid per minute under assay conditions. The pH was modified using Tris-HCl between 7.0 and 9.0 and glycine-NaOH in the range of 9.0–10.0.

Isocitrate dehydrogenase was assayed spectrophotometrically at

*Corresponding author. Fax: (34) (96) 5903464.
E-mail: mjbonete@ua.es

0014-5793/98/$19.00 © 1998 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(98)00911-9

A_{340}, according to the rate of formation of NADPH ($\varepsilon = 6.19 \times 10^3$ M^{-1} cm^{-1}), at 40°C, in 20 mM Tris buffer, pH 8.0; 1mM EDTA; 5 mM $MgCl_2$; 0.22 mM $NADP^+$ and 0.7 mM D,L-isocitrate. The reaction was started by the addition of $NADP^+$. One unit of enzyme activity is defined as the amount of enzyme necessary to produce 1 μmol of NADPH per minute under assay conditions.

Protein concentration was determined as described by Bradford [14], using bovine serum albumin as standard.

2.3. Purification of isocitrate lyase and malate synthase

Isocitrate lyase: *Hf. volcanii* cells were extracted in 50 mM sodium phosphate buffer, pH 6.6; 2.5 M $(NH_4)_2SO_4$; 2 mM DTT. The cell lysate was loaded onto a column of Sepharose-4B equilibrated with the same buffer, and the proteins were eluted with a linear gradient of 2.5–0.5 M $(NH_4)_2SO_4$ (600 ml). The active fractions were pooled and the pool was loaded onto a DEAE-Cellulose column equilibrated with 50 mM sodium phosphate buffer, pH 6.6; 2.5 M $(NH_4)_2SO_4$; 2 mM DTT. The proteins were washed with two column volumes of this buffer and were eluted with 50 mM sodium phosphate buffer, pH 6.6; 2 M NaCl; 2 mM DTT. The active fractions were pooled and loaded onto a Pharmacia FPLC Sephacryl-S300 column equilibrated with 50 mM sodium phosphate buffer, pH 7.3; 2 M NaCl; 2 mM DTT. Proteins were eluted with the same buffer and the active fractions were pooled.

Malate synthase was purified with a three-step procedure, similar to that used in isocitrate lyase purification. The first step (Sepharose-4B chromatography) was the same as that for isocitrate lyase purification (the presence of DTT in extraction buffer and during the purification was not necessary, and it interferes in the enzyme assay). The pool from this column was loaded onto a DEAE-cellulose column equilibrated with 50 mM sodium phosphate buffer, pH 6.6; 2.5 M $(NH_4)_2SO_4$. Proteins were washed with two column volumes of this buffer and were eluted with a linear gradient of 2.5–0 M $(NH_4)_2SO_4$ and 0–2 M NaCl. The active fractions were pooled and loaded onto a Pharmacia FPLC Sephacryl-S300 column equilibrated with 50 mM sodium phosphate buffer, pH 7.3; 2 M KCl. Proteins were eluted with the same buffer and the active fractions were pooled.

2.4. Determination of M_r values

The M_r values for the subunit of isocitrate lyase and malate synthase were determined by SDS-PAGE [15], using standard protein markers (Fig. 1). The M_r values for the native enzymes were determined by gel filtration on a Pharmacia FPLC Sephacryl-S300 column, carried out in 50 mM sodium phosphate buffer, pH 7.3; 2 M NaCl; and 2 mM DTT only when isocitrate lyase M_r was determined, using standards proteins markers (Fig. 2).

3. Results and discussion

3.1. Glyoxylate cycle key enzymes activity

Isocitrate lyase and malate synthase activities were measured in cell extracts of *Hf. volcanii* grown in chemically defined medium [13] with acetate (C2) or lactate (C3) as carbon source. This medium is almost a minimal medium, it only has thiamine and biotin at low concentrations (0.8 and 0.1 μg/ml respectively), apart from salts and the carbon source. This kind of medium is necessary to obtain no interference in the regulation of the enzymes activities due to other carbon sources.

Table 1
Activities in cell extracts of *Hf. volcanii* grown with acetate or with lactate as carbon source

Enzyme	Carbon source	
	Acetate (C2)	Lactate (C3)
Isocitrate lyase	0.14 ± 0.02 (4)	0 (3)
Malate synthase	0.35 ± 0.04 (4)	0 (2)
Isocitrate dehydrogenase	0.7 ± 0.2 (5)	0.5 ± 0.1 (2)

Activities are expressed in U/mg protein: mean value ± standard deviation (in parentheses, number of experiments). *Hf. volcanii* was grown in the chemically defined medium described in Section 2. Isocitrate lyase activity was higher (up to 0.25) when 2 mM DTT was added to the extraction buffer. It was usually not added as it interferes with the malate synthase assay.

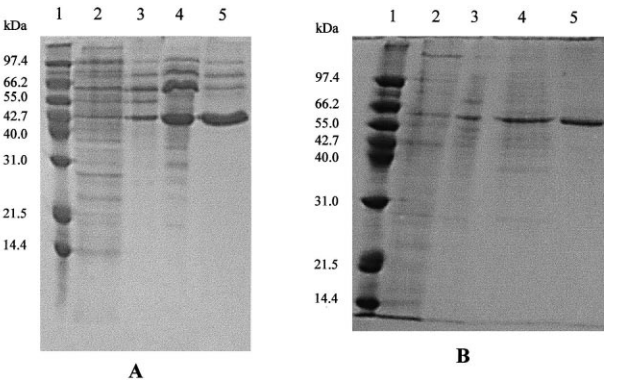

Fig. 1. SDS-PAGE of *Hf. volcanii* isocitrate lyase (A) and malate synthase (B). Lanes 1, standard protein markers; lanes 2, cell extract; lanes 3, protein after chromatography on Sepharose-4B; lanes 4, pool after DEAE-cellulose chromatography; lanes 5, enzyme after gel filtration on FPLC Sephacryl-S300. The M_r values of standard protein markers are indicated.

The activities of glyoxylate cycle key enzymes are necessary to synthesize precursors for carbohydrates only when the carbon source is a C2 compound, such as acetate. Those activities are not needed when the carbon source is a compound with three or more carbons, such as lactate. Results of isocitrate lyase and malate synthase activities in cell extracts of *Hf. volcanii* grown with acetate or with lactate are shown in Table 1. No activity could be detected when lactate was the carbon source, but high activities were measured when acetate was. This shows that the glyoxylate cycle operates in the halophilic archaeon *Hf. volcanii*, and there must be a mechanism of regulation. This regulation of enzyme activities appears in all organisms which have this cycle (regulation of transcription, enzyme modification) [16–21].

Interestingly, citrate synthase from *Hf. volcanii*, an enzyme which operates in the citrate cycle and in the glyoxylate cycle, shows an expression pattern in agreement with the operation of the glyoxylate cycle: when *Hf. volcanii* grows on acetate the level of expression of citrate synthase increases (M.J. Danson, D.G. Maddocks and D.W. Hough, personal communication).

When the glyoxylate cycle is operating, both isocitrate lyase (glyoxylate cycle) and isocitrate dehydrogenase (citrate cycle) compete for isocitrate. To avoid this, in eukaryal organisms these two cycles are physically separated: citrate cycle works at the mitochondrial matrix and glyoxylate cycle does at glyoxysomes [22]. In bacteria, the mechanism of regulation of this branch-point is well studied in *Escherichia coli*: both glyoxylate cycle key enzymes (isocitrate lyase and malate synthase) and isocitrate dehydrogenase are controlled. Genes for isocitrate lyase and malate synthase are in an operon activated by acetate. There is in this operon a gene for an isocitrate dehydrogenase kinase, which inactivates isocitrate dehydrogenase by phosphorylation [16–18].

Because of this, we measured isocitrate dehydrogenase activity in extracts of cells grown with both carbon sources (acetate or lactate). Results are shown in Table 1. We did not find significant differences in *Hf. volcanii* isocitrate dehy-

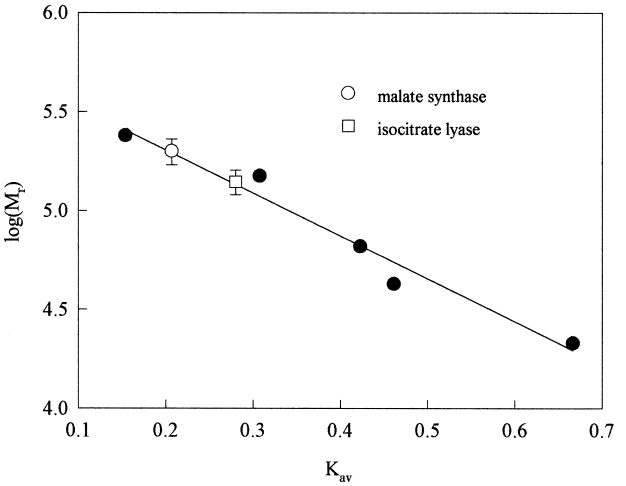

Fig. 2. Determination of the M_r of native glyoxylate cycle key enzymes from *Hf. volcanii* by gel filtration on a Pharmacia FPLC Sephacryl-S300 column. The M_r and K_{av} of standard proteins (●) are, respectively, as follows: trypsin inhibitor (21.5 kDa, 0.67), ovalbumin (42.7 kDa, 0.46), bovine serum albumin (66.2 kDa, 0.42), alcohol dehydrogenase (150 kDa, 0.31) and catalase (240 kDa, 0.15). Values of log(M_r) have been plotted against their K_{av}. The calculated values for isocitrate lyase ($K_{av}=0.28$, 140 ± 20 kDa) and malate synthase ($K_{av}=0.21$, 200 ± 30 kDa) are interpolated.

drogenase activity when glyoxylate cycle is operating or it is not.

3.2. Biochemical characterization of isocitrate lyase and malate synthase

The main characteristics of isocitrate lyase and malate synthase from *Hf. volcanii* are shown in Table 2.

3.3. Isocitrate lyase

The molecular mass of the subunit of isocitrate lyase from *Hf. volcanii* (47 ± 4 kDa) is similar to other bacterial isocitrate lyases [8]. Eukaryal subunits are larger (60–64 kDa), though they show high homology to the sequences of bacterial subunits [23]. Eukaryal subunits of isocitrate lyase have an internal sequence which bacterial subunits lack. Aside from that internal sequence, the homology among all isocitrate lyases is high. The molecular mass of native isocitrate lyase from *Hf. volcanii* (140 ± 20 kDa) suggests that the enzyme is a trimer. Isocitrate lyases are usually homotetramers [8].

The value of the apparent K_m for isocitrate (D-isomer) in the conditions of assay was 1.16 ± 0.04 mM, which is significantly higher than the value obtained for the isocitrate lyase from *Hf. mediterranei* (0.4 mM) [10]. The enzyme showed a narrow temperature optimum for activity at 55°C. Very little

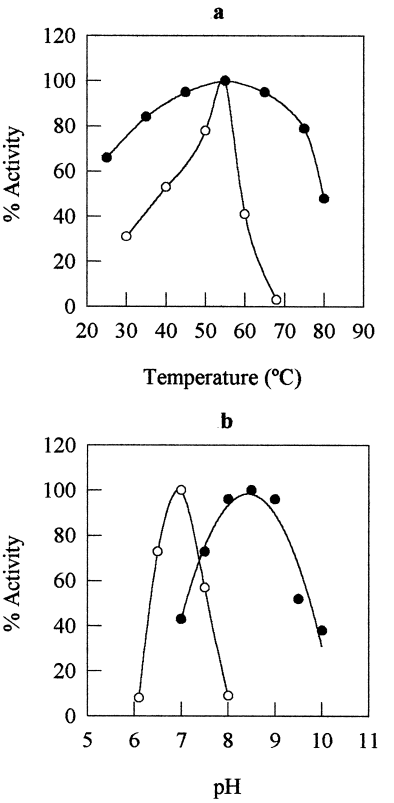

Fig. 3. Temperature (a) and pH (b) dependence of isocitrate lyase and malate synthase activity: (○) isocitrate lyase, (●) malate synthase.

activity was found above 65°C, and the activity at 40°C was 50% of the maximum (Fig. 3). Like isocitrate lyase from *Hf. mediterranei* [10], the enzyme from *Hf. volcanii* has a narrow pH optimum at 7.0. Very little activity was detected at pH 6.1 or 8.0 (Fig. 3).

Isocitrate lyases from different sources require a thiol for their activity in vitro [8]. In [10] this requirement was not detected, probably because activity was measured in cell extracts. Anyway, activity was higher in cell extracts when 2 mM DTT was added to the extraction buffer. We found a reversible loss of activity in isocitrate lyase from *Hf. volcanii* when the enzyme was out of extract without a thiol, such as DTT, which indicates the presence of an active thiol in the enzyme.

3.4. Malate synthase

The molecular mass of the subunit of malate synthase from *Hf. volcanii* (67 ± 4 kDa) is similar to other eukaryal and bacterial subunits [24–26]. The molecular mass of the native

Table 2
Characterization of isocitrate lyase and malate synthase from *Hf. volcanii*

	Isocitrate lyase	Malate synthase
K_m	1.16 ± 0.04 mM (isocitrate)	0.110 ± 0.007 μM (glyoxylate) 0.119 ± 0.008 μM (acetyl-CoA)
Temperature optimum	55°C	50–60°C
pH optimum	7.0	8.0–9.0
Salt optimum	1.5–3.0 M KCl [10]	3.0 M KCl
M_r subunit	47 ± 4 kDa	67 ± 4 kDa
M_r native	140 ± 20 kDa	200 ± 30 kDa
Quaternary structure	Homotrimer	Homotrimer

enzyme (200 ± 30 kDa) suggests that the enzyme is a trimer, like some malate synthases [27]. Other malate synthases have been shown to be dimers or multiaggregates [26,28]. There is another group of bacterial malate synthases that are monomeric enzymes with a larger subunit (around 80 kDa). The sequence of amino acid residues of this group of enzymes displays little homology with other malate synthases [29].

Malate synthase from *Hf. volcanii* required a high salt concentration for activity, the optimum being around 3.0 M KCl. Replacement of KCl by NaCl caused a decrease in activity (about 2-fold). This high salt concentration requirement and the preference for KCl over NaCl is a characteristic of some enzymes from halophilic archaea [30,31], including isocitrate lyase [10]. The apparent K_m for glyoxylate was 0.110 ± 0.007 mM, with 0.2 mM acetyl-CoA, and for acetyl-CoA it was 0.119 ± 0.008 mM, with 0.5 mM glyoxylate. Unlike isocitrate lyase, malate synthase from *Hf. volcanii* showed a broad temperature optimum between 45 and 65°C. The activity of the enzyme at 80°C was half of the maximum (Fig. 3). This enzyme showed a broad optimum pH too, between 8.0 and 9.0, which is higher than the optimum for isocitrate lyase (Fig. 3). The activity of this enzyme is dependent on Mg^{2+}, like all malate synthases.

Acknowledgements: This work was supported by CICYT Grant PB95-0695. J.A. Serrano was the recipient of predoctoral fellowships from Programa Sectorial de Formación de Profesorado Universitario y Personal Investigador. We also thank NATO for a Collaborative Research Grants with D.W. Hough and M.J. Danson (University of Bath, UK).

References

[1] Chistoserdova, L.V. and Lidstrom, M.E. (1996) Microbiology 142, 1459–1468.
[2] Han, L. and Reynolds, K.A. (1997) J. Bacteriol. 179, 5157–5164.
[3] Woese, C.R., Kandler, O. and Wheelis, M.L. (1990) Proc. Natl. Acad. Sci. USA 87, 4576–4579.
[4] Liu, F., Thatcher, J.D. and Epstein, H.F. (1997) Biochemistry 36, 255–260.
[5] Davis, W.L., Goodman, D.B.P., Crawford, L.A., Cooper, O.J. and Matthews, J.L. (1990) Biochim. Biophys. Acta 1051, 276–278.
[6] Popov, V.N., Igamberdiev, A.U. and Volvenkin, S.V. (1996) Biochemistry (Moscow) 61, 1346–1349.
[7] Cioni, M., Pinzauti, G. and Vanni, P. (1981) Comp. Biochem. Physiol. 70B, 1–26.
[8] Vanni, P., Giachetti, E., Pinzauti, G. and McFadden, B.A. (1990) Comp. Biochem. Physiol. 95B, 431–458.
[9] Barns, S.M., Delwiche, C.F., Palmer, J.D. and Pace, N.R. (1996) Proc. Natl. Acad. Sci. USA 93, 9188–9193.
[10] Oren, A. and Gurevich, P. (1995) FEMS Microbiol. Lett. 130, 91–95.
[11] Aitken, D.M. and Brown, A.D. (1969) Biochim. Biophys. Acta 177, 351–354.
[12] Oren, A. and Gurevich, P. (1994) FEMS Microbiol. Ecol. 14, 147–156.
[13] Kauri, T., Wallace, R. and Kushner, D.J. (1990) Syst. Appl. Microbiol. 13, 14–18.
[14] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[15] Laemmli, U. (1970) Nature 227, 680–685.
[16] Sunnarborg, A., Klumpp, D., Chung, T. and LaPorte, D.C. (1990) J. Bacteriol. 172, 2642–2649.
[17] LaPorte, D.C. (1993) J. Cell. Biochem. 51, 14–18.
[18] Chung, T. (1995) Mol. Cells 5, 140–145.
[19] Ordiz, I., Herrero, P., Rodicio, R. and Moreno, F. (1996) FEBS Lett. 385, 43–46.
[20] Mullen, R.T. and Gifford, D.J. (1997) Plant Mol. Biol. 33, 593–604.
[21] Caspary, F., Hartig, A. and Schüller, H.J. (1997) Mol. Gen. Genet. 255, 619–627.
[22] Trelease, R.N., Becker, W.M. and Burke, J.J. (1974) J. Cell. Biol. 60, 483–495.
[23] Rehman, A. and McFadden, B.A. (1996) Arch. Biochem. Biophys. 336, 309–315.
[24] Hartig, A., Simon, M.M., Schuster, T., Daugherty, J.R., Yoo, H.S. and Cooper, T.G. (1992) Nucleic Acids Res. 20, 5677–5686.
[25] Guex, N., Henry, H., Flach, J., Richter, H. and Widmer, F. (1995) Planta 197, 369–375.
[26] Huttner, S., Mecke, D. and Fröhlich, K.U. (1997) Gene 188, 239–246.
[27] Durchschlag, H., Biedermann, G. and Eggerer, H. (1981) Eur. J. Biochem. 114, 255–262.
[28] Henry, H., Escher, C.L. and Widmer, F. (1992) Plant Sci. 82, 21–27.
[29] Molina, I., Pellicer, M.T., Badia, J., Aguilar, J. and Baldoma, L. (1994) Eur. J. Biochem. 224, 541–548.
[30] Kushner, D.J. (1986) FEMS Microbiol. Rev. 39, 121–127.
[31] Camacho, M.L., Brown, R.A., Bonete, M.J., Danson, M.J. and Hough, D.W. (1995) FEMS Microbiol. Lett. 134, 85–90.