Evidence for an operative glyoxylate cycle in the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*

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Abstract Both key enzymes for the glyoxylate cycle, isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), were purified and characterized from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius*. Whereas the former enzyme was copurified with the aconitase, the latter enzyme could be enriched to apparent homogeneity. Amino acid sequencing of three internal peptides of the isocitrate lyase revealed the presence of highly conserved residues. With respect to cofactor requirement and quarternary structure the crenarchaeal malate synthase might represent a novel type of this enzyme family. High activities of both glyoxylate cycle enzymes could already be detected in extracts of glucose grown cells and both increased about two-fold in extracts of acetate grown cells.

Key words: Anaplerotic pathway; Isocitrate lyase; Malate synthase; Isocitrate dehydrogenase; Magnesium dependence

1. Introduction

Sulfolobus acidocaldarius, a thermoacidophilic crenarchaeon, grows optimally at 75–80°C and pH 2–3 under aerobic conditions [1]. The central catabolic carbohydrate metabolism of this archaeon and the closely related *S. solfataricus* consists of a non-phosphorylated Entner–Doudoroff pathway [2–4] and a complete oxidative citric acid cycle was suggested to function in both organisms, as reviewed in [5,6]. In contrast to the well studied pathways for glucose degradation in *Sulfolobus*, nearly nothing is known about gluconeogenesis in this thermoacidophile (for review see [5,6]). Furthermore, no biochemical evidence exists for a glyoxylate cycle in any member of the crenarchaeal branch. However, the presence of both key enzymes of this carbon-conserving bypass of the oxidative tricarboxylic acid cycle was recently demonstrated in the euryarchaeon *Haloferax volcanii* [7] and in cytosolic fractions of other halophilic archaea [8]. In the course of the purification of the aconitase from *S. acidocaldarius*, the enrichment of another protein was observed [9]. N-terminal sequencing of this protein revealed striking homology to a putative isocitrate lyase from *S. solfataricus*. In the following, biochemical data about the characterization of both the first crenarchaeal isocitrate lyase and malate synthase are presented suggesting the presence of a functional glyoxylate cycle in crenarchaea, too.

2. Materials and methods

2.1. Cell growth and preparation of cytosol

S. acidocaldarius cells (DSM 639) were grown in Brocks medium [1], supplemented with 10 mM K_2SO_4, 0.001% yeast extract and either 5 mM glucose or 5 mM acetate as carbon sources. Cell fermentation was performed in a gyratory shaker at 78°C in 5 l conical flasks filled with 2 l growth medium. The cells were harvested in the early stationary phase at an optical density at 546 nm ($OD_{546\,nm}$) of 1.2 or 0.35 for glucose or acetate as carbon sources, respectively, and washed as described [9]. The cells were frozen in liquid nitrogen and stored at −70°C. Typical cell yields were about 1.4 g or 0.3 g wet cells per liter for glucose or acetate grown cells, respectively.

Cells were then disrupted and the cytosolic fraction prepared as described [9].

2.2. Purification of isocitrate lyase and malate synthase

Isocitrate lyase was copurified with the aconitase as described [9]. Malate synthase was purified to apparent homogeneity in a four step procedure.

In the first step, 2 ml of buffer Ia containing 10 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), 2 M $(NH_4)_2SO_4$, 0.5 mM dithiothreitol (DTT), pH 7.0 was added to 2 ml highly concentrated cytosol (c_{Prot} = 46 mg/ml) obtained from glucose grown cells. 2 ml of this mixture was loaded on a Phenyl Sepharose HP column (Amersham Pharmacia Biotech, V = 4 ml) equilibrated with buffer Ib, containing 10 mM Mops, 1 M $(NH_4)_2SO_4$, 0.5 mM DTT, pH 7.0, with a flow rate of 2 ml/min. After washing the column with 20 ml buffer Ib, the bound proteins were eluted with a linear gradient (V = 40 ml) ranging from 1 to 0 M $(NH_4)_2SO_4$ in buffer Ic, containing 10 mM Mops, 0.5 mM DTT, pH 7.0. Malate synthase activity eluted between 0.95 and 0.65 M $(NH_4)_2SO_4$. 2 ml fractions were collected and assayed for malate synthase activity (see below). Fractions with volume activities higher than 0.7 U/ml were pooled. This step was performed twice and the highly active fractions of both runs were combined and concentrated by ultrafiltration with an Ultra RC 10 membrane (Amicon).

In the second step, 2 ml of the Phenyl Sepharose pool (about 9 mg protein) was loaded with a flow rate of 2 ml/min on a gel filtration column (Superdex 200 16/60 prep. grade, Amersham Pharmacia Biotech, V = 125 ml) equilibrated with buffer II, containing 10 mM Tris, 100 mM NaCl, pH 7.5. Fractions with malate synthase activity higher than 1.6 U/ml were pooled and concentrated by ultrafiltration.

In the third step, the concentrated pool fraction (about 0.75 mg protein) was diluted 1:40 with buffer III, containing 10 mM Tris, 2.5 mM $MgCl_2$, pH 8.3 and then concentrated again by ultrafiltration, as described above, to a final volume of 1 ml. Subsequently, 0.5 ml of the sample was loaded with a flow rate of 2 ml/min on a Red-Sepharose column (Reactive red 120 agarose fast flow, Sigma, V = 1 ml), equilibrated with buffer III. Then the column was washed with 5 ml buffer III. The bound proteins were eluted with a linear gradient (V = 15 ml) from 0 to 75% buffer IV, containing 10 mM Tris, 1 M

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Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; Mops, 3-(*N*-morpholino)propanesulfonic acid; ICDH, isocitrate dehydrogenase (EC 1.1.1.42)

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NaCl, 2.5 mM $MgCl_2$, pH 8.3. Most of the malate synthase activity eluted between 75 and 325 mM NaCl. Fractions with activities higher than 0.7 U/ml were pooled. This step was performed twice and the highly active fractions of the two runs were combined and concentrated by ultrafiltration.

In the final step, the concentrated pool fraction was diluted 1:10 with buffer V, containing 10 mM Tris, pH 8.5, and then concentrated again by ultrafiltration to a final volume of 1 ml. The combined sample was loaded with a flow rate of 2 ml/min on an anion-exchange chromatography column (Uno-Q1, Bio-Rad, $V = 1.35$ ml), equilibrated with buffer V. After washing the column with 5 ml buffer V, the bound proteins were eluted with a linear gradient ($V = 15$ ml) from 0 to 25% buffer VI, containing 10 mM Tris, 1 M NaCl, pH 8.5. Malate synthase eluted between 75 and 150 mM NaCl. Fractions with activities higher than 1 U/ml were pooled and concentrated.

2.3. Measurement of enzyme activities

Isocitrate lyase and isocitrate dehydrogenase (ICDH) activities were determined as described [9]. Malate synthase activity was measured according to the rate of formation of 5-mercapto-2-nitrobenzoic acid in the presence of glyoxylate, acetyl-CoA and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The assay was performed in a buffer containing 25 mM Bistris-propane/HCl, 100 mM NaCl, pH 8.0 at 75°C and followed via the increase in absorbance at 412 nm ($\varepsilon = 13\,600$ M^{-1} cm^{-1}). The assay mixture ($V = 1$ ml) contained 0.075 mM DTNB, 0.05–0.1 mM acetyl-CoA and 0.5–20 μl sample. After 1 min of preincubation, the specific reaction was started by adding 1 mM glyoxylate. One unit of enzyme activity is defined as the amount of enzyme to produce 1 μmol of 5-mercapto-2-nitrobenzoic acid per minute.

Determination of the respective K_M constants was performed for isocitrate lyase and ICDH in the range from 15.6 μM to 4 mM isocitrate. For malate synthase, the range of substrate concentration varied from 1 to 500 μM acetyl-CoA in the presence of 500 μM glyoxylate or from 15.6 μM to 1 mM glyoxylate in the presence of 100 μM acetyl-CoA. Determination of the $K_{I\,(50\%)}$ values was performed by varying the respective inhibitor concentration at a fixed concentration of isocitrate (2.5 mM).

2.4. Analytical gel filtration

Determination of the M_r values for the purified, native proteins was performed on a Superdex 200 16/60 column (Amersham Pharmacia Biotech) equilibrated with a buffer containing 20 mM Tris–HCl, 1 mM DTT, 1 mM citrate, 250 mM NaCl, pH 7.6. The column was calibrated with the following proteins from *S. acidocaldarius*: malate dehydrogenase (134 kDa) [10], ICDH (96 kDa) [11], citrate synthase (83 kDa) [12] and fumarate hydratase (192 kDa) [13]. Flow rate was 2 ml/min. The proteins were detected by absorption at 278 nm and fractions of 2 ml were screened for the respective enzymatic activities.

2.5. Other methods

Protein was determined by the Bio-Rad system based on the Lowry method [14]. SDS–PAGE was carried out according to Laemmli [15] on 15% and 12.5% (w/v) gels with an acrylamide/bisacrylamide ratio of 100:1. Proteins and peptides were visualized by Coomassie blue staining. In gel cleavage of isocitrate lyase and peptide sequencing were performed as described [9]. Primary structures of the peptides were compared with respective sequences in the available databases by BLAST search [16]. The fragments were further aligned by using the program CLUSTAL X (version 1.64b) [17].

2.6. Materials

Materials for chromatography were purchased from Amersham Pharmacia Biotech and Sigma. Chemicals were from Sigma, Serva, ICN, Gerbu and Fermentas.

3. Results and discussion

3.1. Biochemical characterization of isocitrate lyase

The copurification of both aconitase and isocitrate lyase has already been reported by us [9]. As determined by SDS–PAGE the apparent molecular weights were 96 kDa for the aconitase and 46 kDa for the isocitrate lyase (see Fig. 1A).

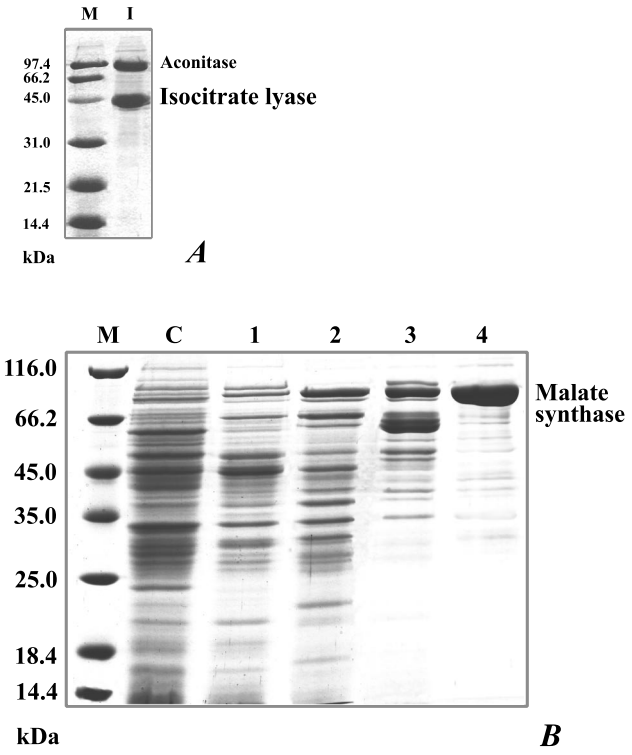

Fig. 1. SDS–PAGE of the final stage of isocitrate lyase/aconitase preparation (A) and fractions of subsequent steps of malate synthase purification (B). A: 15% PAA. Lane M: molecular mass marker proteins (phosphorylase b: 97.4 kDa; bovine serum albumin (BSA): 66.2 kDa; ovalbumin: 45.0 kDa; carbonic anhydrase: 31.0 kDa; soybean trypsin inhibitor: 21.5 kDa; lysozyme: 14.4 kDa). Lane I: pool after affinity chromatography. Protein was 12.5 μg, except for marker lane. B: 12.5% PAA. Lane M: molecular mass marker proteins (β-galactosidase: 116 kDa; BSA: 66.2 kDa; ovalbumin: 45.0 kDa; lactate dehydrogenase: 35 kDa; *Bsp*98I (restriction endonuclease 98 isoschizomer from *Bacillus subtilis*): 25 kDa; β-lactoglobulin: 18.4 kDa; lysozyme: 14.4 kDa). Lane C: cytosol; lane 1: pool after hydrophobic interaction chromatography; lane 2: pool after preparative gel exclusion chromatography; lane 3: pool after affinity chromatography; lane 4: pool after anion-exchange chromatography. Protein was 8 μg, except for lane C (20 μg) and marker lane.

These data agree well with the calculated masses for the putative proteins from *S. solfataricus* with 95.8 and 48.9 kDa, respectively [18]. The enzymes could not be separated by analytical gel filtration and an apparent molecular mass of 145 ± 15 kDa was estimated for the native proteins by using standard calibration proteins from mesophilic species [9]. However, recently an irregular mobility in gel exclusion chromatography experiments has been observed for certain cytosolic proteins of *S. acidocaldarius* [19,20]. Therefore, endogenous enzymes were used as internal calibration standards and an apparent molecular mass of 215 ± 25 kDa could be estimated for the copurified proteins. This result indicates a homodimeric structure of the aconitase and a homotetrameric crenarchaeal isocitrate lyase, which is in line with the quarternary structures of most bacterial and eukaryal isocitrate lyases (for review see [21]) and also for the other archaeal enzyme from *H. volcanii* [22].

Enzymatic analysis clearly indicated the 46 kDa protein to function as an isocitrate lyase, catalyzing the cleavage of isocitrate to glyoxylate and succinate. The enzyme could be enriched about 30-fold to a final specific activity of 7.9 U/mg.

Table 1
Characterization of isocitrate lyase and malate synthase from *S. acidocaldarius*

	Isocitrate lyase	Malate synthase
Overall purification	30	139
Specific activity of purified enzyme (U/mg)	7.9	57.0
Temperature optimum	80°C	79.5°C
Activation energy (kJ/mol)	56.7 ± 3.2	36.9 ± 2.5
pH optimum	6.0	6.8
K_M (μM)	720 ± 150 (isocitrate)	44 ± 3.3 (glyoxylate) 5.6 ± 0.9 (acetyl-CoA)
$K_{I\,(50\%)}$ (μM)	70 (itaconate) 15 (3-nitro-propionate)	n.d.
Mg^{2+} dependence	yes	no
M_r subunit (kDa)[a]	46	88.4 ± 7.3
M_r native (kDa)[b]	215 ± 25	206 ± 25
Quarternary structure	homotetramer	homodimer

[a]Determined by SDS–PAGE.
[b]Determined by gel exclusion chromatography.

Despite of the use of argon-saturated buffers and DTT to overcome the known oxygen sensitivity of isocitrate lyases (see [21]), a significant part of the crenarchaeal protein became irreversibly damaged during the purification procedure. The activity of the enzyme from *S. acidocaldarius*, like known isocitrate lyases, was strictly dependent on Mg^{2+}, with an optimum around 1 mM (Table 1). The temperature optimum for the reaction was approximately 80°C (Fig. 2A), coinciding with the optimum growth temperature of this organism [1]. The activation energy was calculated from an Arrhenius plot as 56.7 ± 3.2 kJ/mol, which is in the typical range of enzymatic reactions (20–80 kJ/mol) (not shown). The pH optimum of the isocitrate lyase around 6.0 (Fig. 2B) was slightly below the internal pH value in *S. acidocaldarius* of 6.5 [23]. Under physiological pH and temperature conditions, i.e. pH 6.5, 80°C and in the presence of 100 mM NaCl, the K_M value for isocitrate was estimated as 720 ± 150 μM for the purified enzyme (Table 1). This result is in the same range as either the reported constants for the two archaeal isocitrate lyases of *H. volcanii* and *H. mediterranei* with 1.16 mM and 400 μM, respectively [7,24], or those obtained for the enzymes of mesophilic Bacteria, e.g. *Corynebacterium glutamicum* and *Escherichia coli* [25,26]. Like other isocitrate lyases, the thermophilic enzyme is significantly inhibited by the succinate analogues itaconate and 3-nitropropionate. The $K_{I\,(50\%)}$ values in the presence of 2.5 mM D,L-isocitrate were determined as 70 and 15 μM, respectively.

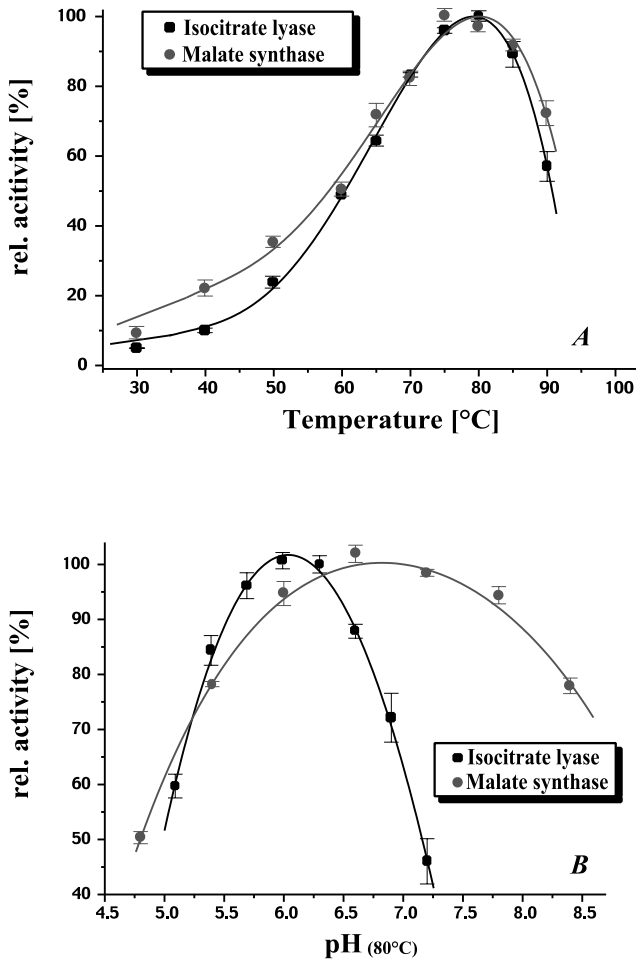

Fig. 2. Temperature (A) and pH (B) dependence of isocitrate lyase and malate synthase activity: (■) isocitrate lyase, (●) malate synthase. Means ± S.D. of at least three different measurements are shown.

BLAST searches of the N-terminus of the isocitrate lyase of *S. acidocaldarius* against the known databases revealed only striking similarity to that of the putative enzyme of *S. solfataricus* [18] (Fig. 3A). To further characterize the protein of *S. acidocaldarius* on the molecular level, the enzyme was cleaved in gel by cyanbromide and the resulting fragments were analyzed by Edman degradation. Alignments of three internal peptides obtained from the 46 kDa protein with the corresponding sequences of the putative isocitrate lyase of *S. solfataricus* and known enzymes from the other two urkingdoms of life, are displayed in Fig. 3B. These results clearly underline that the 46 kDa band corresponds to the first characterized crenarchaeal isocitrate lyase. Surprisingly, many residues in the respective sequences are found to be strictly conserved not only across domains (eukaryal, bacterial and archaeal), but also across physical (mesophilic and thermophilic) boundaries. The N-terminal lysine, the cysteine and the histidine of the highly conserved pentapeptide located in peptide 2 are implicated to be directly involved in catalysis as, for example, in the case of the isocitrate lyase of *E. coli* [27–29]. At least the latter two residues might be critical also in the catalytic process of the archaeal protein, since the purified enzyme was rapidly and completely inactivated in the presence of either the thiol group or the histidine modifying reagents, DTNB and dimethyl pyrocarbonate, respectively. Interestingly, with

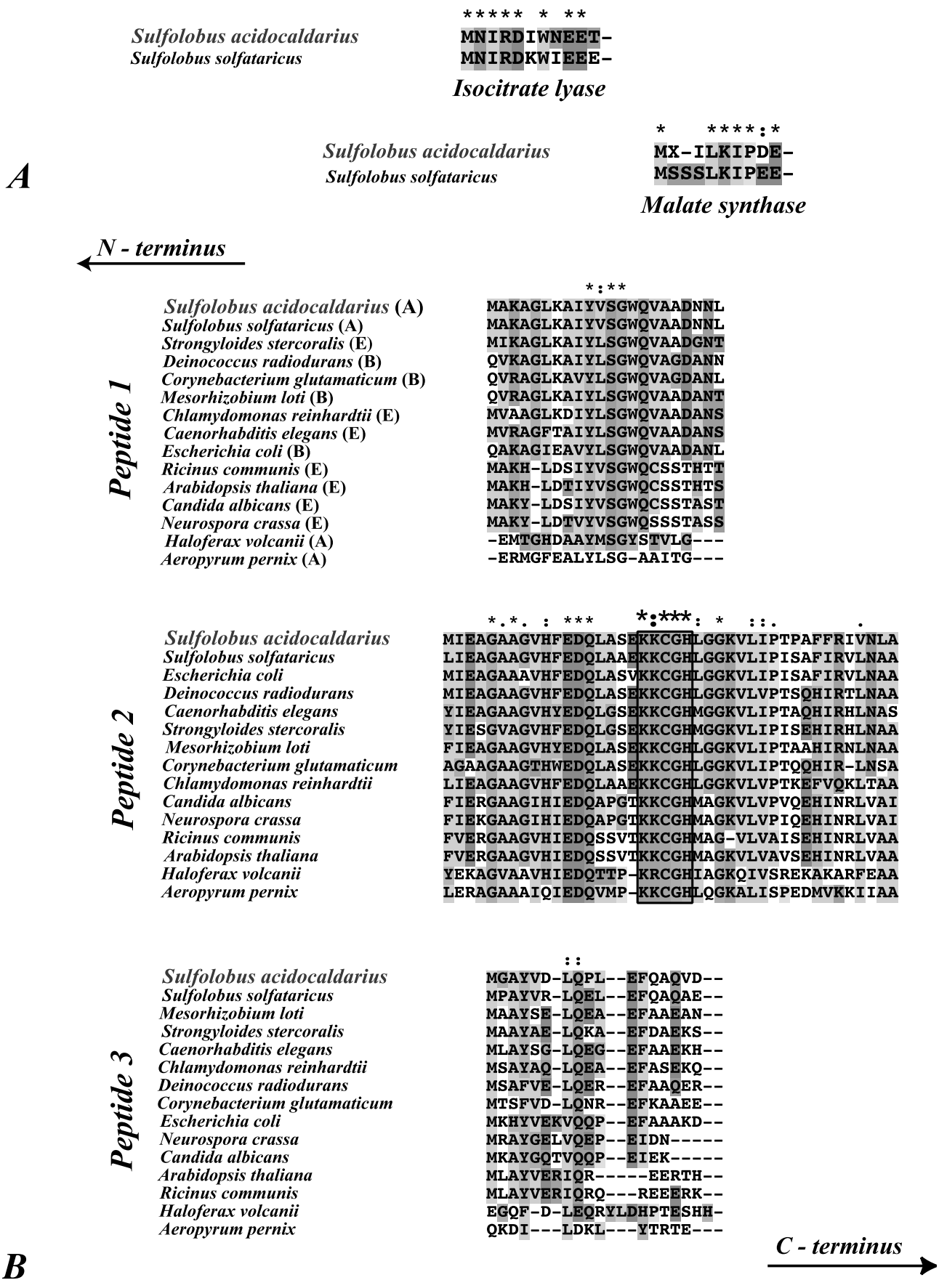
***** * **
Sulfolobus acidocaldarius MNIRDIWNEET-
Sulfolobus solfataricus MNIRDKWIEEE-
Isocitrate lyase
A
* ****:*
Sulfolobus acidocaldarius MX-ILKIPDE-
Sulfolobus solfataricus MSSSLKIPEE-
Malate synthase
N - terminus
Peptide 1
*:**
Sulfolobus acidocaldarius (A) MAKAGLKAIYVSGWQVAADNNL
Sulfolobus solfataricus (A) MAKAGLKAIYVSGWQVAADNNL
Strongyloides stercoralis (E) MIKAGLKAIYLSGWQVAADGNT
Deinococcus radiodurans (B) QVKAGLKAIYLSGWQVAGDANN
Corynebacterium glutamaticum (B) QVRAGLKAVYLSGWQVAGDANL
Mesorhizobium loti (B) QVRAGLKAIYLSGWQVAADANT
Chlamydomonas reinhardtii (E) MVAAGLKDIYLSGWQVAADANS
Caenorhabditis elegans (E) MVRAGFTAIYLSGWQVAADANS
Escherichia coli (B) QAKAGIEAVYLSGWQVAADANL
Ricinus communis (E) MAKH-LDSIYVSGWQCSSTHTT
Arabidopsis thaliana (E) MAKH-LDTIYVSGWQCSSTHTS
Candida albicans (E) MAKY-LDSIYVSGWQCSSTAST
Neurospora crassa (E) MAKY-LDTVYVSGWQSSSTASS
Haloferax volcanii (A) -EMTGHDAAYMSGYSTVLG---
Aeropyrum pernix (A) -ERMGFEALYLSG-AAITG---
Peptide 2
.. : *** *:***: * ::. .
Sulfolobus acidocaldarius MIEAGAAGVHFEDQLASEKKCGHLGGKVLIPTPAFFRIVNLA
Sulfolobus solfataricus LIEAGAAGVHFEDQLAAEKKCGHLGGKVLIPISAFIRVLNAA
Escherichia coli MIEAGAAAVHFEDQLASVKKCGHLGGKVLIPISAFIRVLNAA
Deinococcus radiodurans MIEAGAAGVHFEDQLASEKKCGHLGGKVLVPTSQHIRTLNAA
Caenorhabditis elegans YIEAGAAGVHYEDQLGSEKKCGHMGGKVLIPTAQHIRHLNAS
Strongyloides stercoralis YIESGVAGVHFEDQLGSEKKCGHMGGKVLIPISEHIRHLNAA
Mesorhizobium loti FIEAGAAGVHYEDQLASEKKCGHLGGKVLIPTAAHIRNLNAA
Corynebacterium glutamaticum AGAAGAAGTHWEDQLASEKKCGHLGGKVLIPTQQHIR-LNSA
Chlamydomonas reinhardtii LIEAGAAGVHFEDQLAAEKKCGHLGGKVLVPTKEFVQKLTAA
Candida albicans FIERGAAGIHIEDQAPGTKKCGHMAGKVLVPVQEHINRLVAI
Neurospora crassa FIEKGAAGIHIEDQAPGTKKCGHMAGKVLVPIQEHINRLVAI
Ricinus communis FVERGAAGVHIEDQSSVTKKCGHMAG-VLVAISEHINRLVAA
Arabidopsis thaliana FVERGAAGVHIEDQSSVTKKCGHMAGKVLVAVSEHINRLVAA
Haloferax volcanii YEKAGVAAVHIEDQTTP-KRCGHIAGKQIVSREKAKARFEAA
Aeropyrum pernix LERAGAAAIQIEDQVMP-KKCGHLQGKALISPEDMVKKIIAA
Peptide 3
::
Sulfolobus acidocaldarius MGAYVD-LQPL--EFQAQVD--
Sulfolobus solfataricus MPAYVR-LQEL--EFQAQAE--
Mesorhizobium loti MAAYSE-LQEA--EFAAEAN--
Strongyloides stercoralis MAAYAE-LQKA--EFDAEKS--
Caenorhabditis elegans MLAYSG-LQEG--EFAAEKH--
Chlamydomonas reinhardtii MSAYAQ-LQEA--EFASEKQ--
Deinococcus radiodurans MSAFVE-LQER--EFAAQER--
Corynebacterium glutamaticum MTSFVD-LQNR--EFKAAEE--
Escherichia coli MKHYVEKVQQP--EFAAAKD--
Neurospora crassa MRAYGELVQEP--EIDN-----
Candida albicans MKAYGQTVQQP--EIEK-----
Arabidopsis thaliana MLAYVERIQR-----EERTH--
Ricinus communis MLAYVERIQRQ---REEERK--
Haloferax volcanii EGQF-D-LEQRYLDHPTESHH-
Aeropyrum pernix QKDI---LDKL---YTRTE---
C - terminus
B

Fig. 3. A: Comparison of N-terminal amino acid sequences of *S. acidocaldarius* isocitrate lyase and malate synthase with the putative enzymes of *S. solfataricus*. B: Alignments of three internal peptides of isocitrate lyase from *S. acidocaldarius* obtained by CNBr cleavage with selected sequences of putative and known isocitrate lyases. The sequence of *A. pernix* belongs to a putative carboxy phosphonoenolpyruvate phosphonomutase. A highly conserved pentapeptide, found in nearly all isocitrate lyases, is boxed in peptide 2. Identical residues between all sequences are indicated with an asterisk; dots indicate conservative (:) and similar (.) amino acid replacements, respectively. (A) Archaea; (B) Bacteria; (E) Eukarya.
←

exception of the putative protein from *S. solfataricus*, inspection of the published archaeal genomes did not reveal any other homologues of known isocitrate lyases in the third domain of life. The sequence of the thermophilic crenarchaeon *Aeropyrum pernix* [30] belongs to a putative carboxy phosphonoenolpyruvate phosphonomutase (EC 2.7.8.23), which is also annotated in the genomes of all other aerobic and thermophilic archaea published, thus far [18,31–33]. Carboxy phosphonoenolpyruvate phosphonomutases, catalyzing the carbon–phosphorus bond formation in the synthesis of the herbicide bialophos [34], share sequence homologies to isocitrate lyases, but exhibit significantly lower molecular weights. Interestingly, the euryarchaeal isocitrate lyase from *H. volcanii* [22] shows a higher similarity to the latter proteins than to the fragments of either the enzyme of *S. acidocaldarius* or the putative isocitrate lyase from *S. solfataricus*.

3.2. Biochemical characterization of malate synthase

The second key enzyme of the glyoxylate cycle, malate synthase, catalyzing the condensation reaction of glyoxylate and acetyl-CoA to malate, could be enriched about 140-fold, based on enzymatic activity, as shown in Table 1. The enzyme turned out to be highly substrate specific. Whereas the relative activity of the enzyme with oxaloacetate is about 10% of the maximum achieved with glyoxylate, less than 5% could be detected with 2-oxoglutarate and pyruvate as substrates. Like the isocitrate lyase, the crenarchaeal malate synthase is well adopted to the thermophilic growth conditions of *S. acidocaldarius*. The temperature optimum for the reaction was determined at 80°C and the pH optimum, around 6.8, is only slightly above the internal pH value of this organism (Fig. 2A,B). The activation energy of the reaction was calculated from an Arrhenius diagram as 36.9 ± 2.5 kJ/mol, which is also in the typical range of enzymatic reactions (20–80 kJ/mol) (Table 1). For the purified enzyme, the K_M values for glyoxylate and acetyl-CoA could be estimated as 44 ± 3.3 μM and 5.6 ± 0.9 μM, respectively (Table 1). Whereas the former constant is in the range of the respective value for the halobacterial enzyme (110 μM), the latter is significantly lower in the crenarchaeal enzyme as compared to the euryarchaeal one (119 μM) [7]. But both K_M values agree well with the respective constants determined for malate synthases from yeast or *C. glutamaticum* [35,36]. In striking contrast to the euryarchaeal and other known malate synthases, the crenarchaeal enzyme showed no dependence on Mg^{2+} ([7], for a review see [37]). The protein of *S. acidocaldarius* could be purified to nearly homogeneity, as shown in Fig. 1. Based on its N-terminal sequence (see Fig. 3A) and the apparent molecular mass of the subunit (88.5 ± 7.0 kDa compared to 94 kDa), the malate synthase of *S. acidocaldarius* turned out to be very similar to the putative enzyme of *S. solfataricus* [18]. But obviously the enzyme of *S. acidocaldarius* differs significantly from the protein of *H. volcanii*. Whereas by gel exclusion chromatography the relative molecular mass of the native crenarchaeal malate synthase could be estimated as 206 ± 25 kDa, indicating a functional homodimer, the euryarchaeal enzyme was characterized as functional tetramer with a subunit size around 48 kDa [22]. Based on sequence alignments, the euryarchaeal enzyme forms a novel class of malate synthases [22]. Also the *Sulfolobus* enzyme differs remarkably from both the class A malate synthases, present in Eukarya and Bacteria, and the class G enzymes, exclusively found in the bacterial domain. The molecular mass around 90 kDa of the crenarchaeal subunit is significantly larger than that of the class A enzymes, which are in the range from 60 to 65 kDa (for review see [37]). Despite a similar subunit size, the crenarchaeal malate synthase also is different from the class G enzymes with respect to quarternary structure and cofactor requirement, as the latter enzymes were all described as monomers (M_r around 80 kDa), with an absolute need for divalent metal ions for catalytic activity [35,38].

3.3. Glyoxylate cycle enzyme activity under different growth conditions

Aerobic growth of many microorganisms in the presence of acetate or C_2-providing compounds, e.g. fatty acids, as major carbon sources, depends on a functional glyoxylate cycle [39]. Thus far, acetate has been described to be inhibiting for *S. acidocaldarius* and cellular growth of this thermoacidophilic crenarchaeon seemed to be restricted to substrates, providing at least three carbon atoms [40,41]. Up to a concentration of 5 mM acetate as sole carbon source, however, we were able to grow *S. acidocaldarius* to an $OD_{546\,nm}$ of 0.35 after a cultivation time of about 120 h, indicating a functional glyoxylate cycle in this crenarchaeon. Other C_2 compounds like glycine, glycolate or glyoxylate, as well as β-hydroxybutyrate, a precursor form of fatty acids, failed to support growth or even turned out to be toxic for *S. acidocaldarius*, e.g. glyoxylate.

Whereas in many microorganisms, including *H. volcanii* [7], isocitrate lyase as well as malate synthase activity are generally restricted to cells, growing on C_2 compounds, high specific activities of both glyoxylate cycle key enzymes could already be detected in cytosolic extracts of glucose grown *S. acidocaldarius* cultures. Surprisingly, the specific activities increased only about two-fold in cells, cultivated in the presence of acetate as sole carbon source (Fig. 4). This is in drastic contrast to the situation in e.g. *C. glutamaticum*, where an about 100-fold induction of isocitrate lyase activity and an about 45-fold induction of malate synthase activity were observed under comparable conditions [42]. As recently high acetate concentrations (up 3 mM) were observed in glucose consuming *S. acidocaldarius* cells [4], our data might suggest that in this organism the activities of both glyoxylate cycle enzymes are not mainly triggered by exogenous but by internal produced acetate. In enteric Bacteria, growing on acetate as the sole carbon source, the significantly higher affinity of ICDH, which competes with isocitrate lyase for the same substrate, is reduced by post-translational phosphorylation (for a

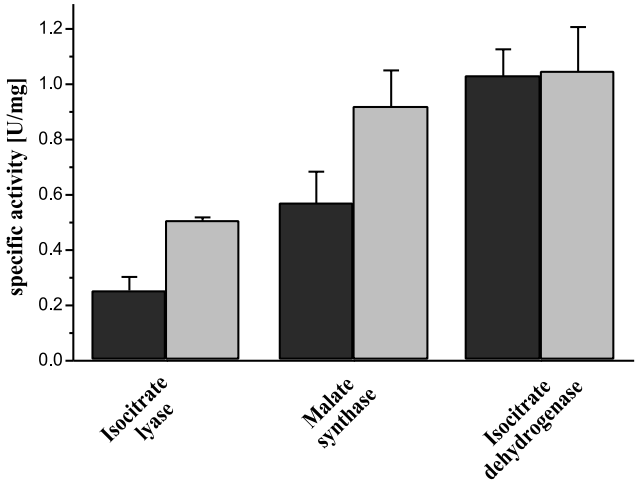

Fig. 4. Enzymatic activities in cytosolic extracts of *S. acidocaldarius* grown with different carbon sources (black bars: glucose; gray bars: acetate). The mean values ± S.D. of at least three independent cultures are shown.

review see [43]). The ICDH of *S. acidocaldarius* also exhibits a drastically lower K_M (29 ± 3 μM) for isocitrate, when measured under the same conditions as described for the endogenous isocitrate lyase (see above). But strikingly, the specific activity of the former enzyme remained apparently the same in either acetate or glucose grown cells, as shown in Fig. 4. This observation is in line with former results, where no additional protein phosphorylation could be detected in *S. acidocaldarius*, cultivated in the presence of acetate [40].

As yet, it is beyond the scope of this paper to discuss the nature and level of regulation at the branching point between the final steps of oxidative hexose degradation and the potential starting sequence of gluconeogenesis in *S. acidocaldarius*. However, the presented data indicate for the first time the existence of a functional glyoxylate cycle in the crenarchaeal subdomain of life. Further investigations to clarify the molecular details of its control are in progress.

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