Malate synthase from *Corynebacterium glutamicum*: sequence analysis of the gene and biochemical characterization of the enzyme

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Malate synthase is one of the key enzymes of the glyoxylate cycle and is essential for growth on acetate as sole carbon source. The *aceB* gene from *Corynebacterium glutamicum*, encoding malate synthase, was isolated, subcloned and expressed in *Escherichia coli* and *C. glutamicum*. Sequencing of a 3024 bp DNA fragment containing the *aceB* gene revealed that it is located close to the isocitrate lyase gene *aceA*. The two genes are separated by 597 bp and are transcribed in divergent directions. The predicted *aceB* gene product consists of 739 amino acids with an M_r of 82 362. Interestingly, this polypeptide shows only weak identity with malate synthase polypeptides from other organisms and possesses an extra N-terminal sequence of about 170 amino acid residues. Inactivation of the chromosomal *aceB* gene led to the absence of malate synthase activity and to the inability to grow on acetate, suggesting that only one malate synthase is present in *C. glutamicum*. The malate synthase was purified from an *aceB*-overexpressing *C. glutamicum* strain and biochemically characterized. The native enzyme was shown to be a monomer migrating at an M_r of about 80 000. By sequencing the N-terminus of malate synthase the predicted translational start site of the enzyme was confirmed. The enzyme displayed K_m values of 30 μ M and 12 μ M for the substrates glyoxylate and acetyl CoA, respectively. Oxalate, glycolate and ATP were found to be inhibitors of malate synthase activity. The present study provides evidence that the malate synthase from *C. glutamicum* is functionally similar to other malate synthase enzymes but is different both in size and primary structure.

Keywords: *Corynebacterium glutamicum*, acetate metabolism, glyoxylate cycle, malate synthase, isocitrate lyase

INTRODUCTION

Utilization of acetate or fatty acids as sole carbon sources requires the operation of the glyoxylate cycle as an anaplerotic pathway for replenishing C4 molecules of the tricarboxylic acid (TCA) cycle (Kornberg, 1966). Key enzymes of the glyoxylate cycle are isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), which bypass the decarboxylation steps of the TCA cycle. Isocitrate lyase cleaves isocitrate to give succinate and glyoxylate and malate synthase catalyses the irreversible

aldol condensation of glyoxylate and acetyl CoA to form malate and CoA.

Malate synthase has been biochemically characterized from a variety of organisms (reviewed by Cioni *et al.*, 1981), including some bacteria such as *Escherichia coli*, *Bacillus stearothermophilus* and *Pseudomonas ovalis* (Chell & Sundaram, 1975, 1978; Falmagne & Wiame, 1973; Dixon *et al.*, 1960). The properties of the purified malate synthases are similar with respect to K_m values, specific activity, inhibitors and requirement of Mg^{2+} for activity. The prokaryotic enzymes are monomers ($M_r \approx 60\,000$) whereas the eukaryotic malate synthases are homomultimers with subunit sizes corresponding to the prokaryotic enzymes. Besides biochemical characterization of the enzyme, genes coding for malate synthase have been

Abbreviation: WT, wild-type.

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cloned and sequenced from *E. coli* (Maloy & Nunn, 1982; Byrne *et al.*, 1988) and from several eukaryotes, e.g. *Saccharomyces cerevisiae*, *Neurospora crassa*, *Aspergillus nidulans*, *Brassica napus*, *Candida tropicalis* and cucumber (Hartig *et al.*, 1992; Fernandez *et al.*, 1993; Thomas *et al.*, 1988; Sandemann *et al.*, 1991; Comai *et al.*, 1989; Hikida *et al.*, 1991; Graham *et al.*, 1989). The genes encoding malate synthase are generally only expressed during growth on acetate, glyoxylate or compounds which are metabolized to acetate, acetyl CoA or glyoxylate, e.g. fatty acids, ethanol or allantoin (Kornberg, 1966; Armitt *et al.*, 1976; Sandemann & Hynes, 1989; Hikida *et al.*, 1991; Hartig *et al.*, 1992; Fernandez *et al.*, 1993). In *E. coli*, the malate synthase gene *aceB* was found to be organized in an operon together with the *aceA* gene encoding isocitrate lyase and the *aceK* gene coding for isocitrate dehydrogenase-kinase/phosphatase, which is involved in the regulation of isocitrate dehydrogenase (Brice & Kornberg, 1968; Maloy & Nunn, 1982; Chung *et al.*, 1988; LaPorte *et al.*, 1985).

Corynebacterium glutamicum is a Gram-positive organism widely used in the industrial production of primary metabolites, e.g. amino acids (Liebl, 1991). The organism is able to grow on acetate and, accordingly, both isocitrate lyase and malate synthase activities are present in it (Kinoshita, 1985). It has been shown that acetate also serves as substrate for the production of amino acids such as glutamate, lysine and threonine (reviewed in Kinoshita & Tanaka, 1972). In some production strains, the formation of threonine was even higher in the presence of acetate than on glucose minimal medium (Akashi *et al.*, 1979), suggesting that the glyoxylate cycle may be an important target in the genetic construction of defined amino-acid-producing strains. Therefore, we initiated genetic and biochemical studies on both the isocitrate lyase and the malate synthase of *C. glutamicum*. The isocitrate lyase has recently been biochemically characterized and the respective *aceA* gene has been cloned and sequenced (Reinscheid *et al.*, 1994). As in *E. coli*, expression of the *aceA* gene was found to be tightly regulated; however, several lines of evidence suggested that in *C. glutamicum* the *aceA* gene is not transcriptionally linked to the *aceB* gene and, thus, the two genes have an organization different from that in *E. coli*. In the present study we describe the cloning of the *C. glutamicum* malate synthase gene *aceB*, its nucleotide sequence and the deduced amino acid sequence. We show data about the homologous and heterologous expression of *aceB* and its chromosomal inactivation in *C. glutamicum* and, finally, we present the purification and biochemical analysis of the malate synthase enzyme.

METHODS

Bacteria, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. M9 medium (Sambrook *et al.*, 1989) was used as minimal medium for *E. coli*; the minimal medium used for *C. glutamicum* has been described previously (Eikmanns *et al.*, 1991b) and contained 4% (w/v) glucose, 2% (w/v) acetate, or 1% (w/v) glucose plus 1% (w/v) acetate. LB medium (Sambrook *et al.*, 1989) was used as

the complex medium for both organisms. For the growth of *E. coli* DV21A05 on glucose minimal medium, succinate was added at 15 mM. When appropriate, ampicillin (100 µg ml⁻¹) or kanamycin (50 µg ml⁻¹) was added to the medium. Both organisms were grown under aerobic conditions, *E. coli* at 37 °C, *C. glutamicum* at 30 °C.

DNA preparation and transformation. Plasmids from *E. coli* were isolated by the method of Birnboim (1983). Chromosomal DNA from *C. glutamicum* was obtained as described previously (Eikmanns, 1992). Transformation of *C. glutamicum* was performed by electroporation (Liebl *et al.*, 1989) and *E. coli* was transformed by the CaCl₂ method (Sambrook *et al.*, 1989).

DNA manipulations. All restriction enzymes, T4 DNA ligase, Klenow polymerase and calf intestine phosphatase were obtained from Boehringer Mannheim and used as instructed by the manufacturer. Restriction-generated fragments were separated on 0.8% agarose gels and isolated and purified by using the GeneClean kit (Dianova; Bio 101).

DNA hybridization experiments were performed as described previously (Reinscheid *et al.*, 1994). The 0.84 kb *SmaI*-*KpnI* fragment isolated from plasmid pEKB1 and the 0.92 kb *Asp700*-*BfrI* fragment isolated from plasmid pEKA2 were labelled with digoxigenin-dUTP and used as probes.

For sequencing, the 1.61 kb *BfrI*-*KpnI* fragment and the 1.79 kb *HindIII*-*PvuI* fragment from plasmid pAB-17 were blunt-ended and ligated into plasmid pUC18. From the resulting plasmids progressive unidirectional deletions of the inserted DNA were created using the Erase-a-base kit from Promega. Sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the AutoRead sequencing kit from Pharmacia with subsequent electrophoretic analysis with an A.L.F. DNA sequencer from Pharmacia. Sequence data were compiled and analysed by the HUSAR program package from EMBL.

Gene disruption. Gene disruption was performed as described previously by Schwarzer & Pühler (1991). An *aceB* internal 0.42 kb *SphI*-*KpnI* fragment was blunt-ended and ligated into the *ScaI* site of the mobilizable *E. coli* vector pSUP301, which is nonreplicative in *C. glutamicum*. The resulting plasmid was introduced into *C. glutamicum* via conjugation from *E. coli* S17-1. The conjugation was performed as described by Schäfer *et al.* (1990); the transconjugants were selected on LB agar plates containing kanamycin (15 µg ml⁻¹) and nalidixic acid (50 µg ml⁻¹).

Malate synthase assay. To determine malate synthase activity, cells were grown in 60 ml medium in 500 ml baffled Erlenmeyer flasks to the exponential growth phase, washed twice in 20 ml 50 mM Tris/HCl buffer, pH 7.6, and resuspended in 1 ml of the same buffer. Cells were disrupted by sonication with a microtip-equipped sonifier (Branson) at maximum settings for 10 min (*C. glutamicum*) or for 2 min (*E. coli*) at 0 °C. After centrifugation for 30 min at 13000 g the supernatant was used as crude extract. The protein concentration was determined by the Biuret method (Gornall *et al.*, 1949) using bovine serum albumin as standard. Malate synthase was assayed photometrically at 232 nm and 30 °C according to a modified method described by Dixon & Kornberg (1959). In a final volume of 1 ml, each assay contained 50 mM Tris/HCl, pH 7.6, 40 mM MgCl₂, 0.24 mM acetyl CoA, 2 mM sodium glyoxylate and malate synthase or crude extract. One unit (U) of activity corresponds to 1 µmol malate formed per min. Michaelis constants were obtained by using a Lineweaver-Burk double-reciprocal plot and inhibition constants were determined from linear replots of Lineweaver-Burk slopes versus inhibitor concentrations (Segel, 1975).

Purification of malate synthase. Crude extracts for purification

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics*	Source/reference
Strains		
<i>E. coli</i> DV21A05†	<i>lacZ43</i> , <i>relA1</i> , (<i>speC-glc</i>)63, <i>spoT1</i> , <i>ppc-2</i> , <i>thi-1</i> , <i>aceB6</i> , Hfr	Vanderwinkel & De Vlieghere (1968)
<i>E. coli</i> S17-1	Mobilizing donor strain	Simon <i>et al.</i> (1983)
<i>C. glutamicum</i> WT	WT strain ATCC 13032	American Type Culture Collection
Cosmids/plasmids		
pHC79	Ap ^R Tet ^R	Hohn & Collins (1980)
pHC79-based gene library	<i>C. glutamicum</i> chromosomal DNA cloned in cosmid pHC79	Börmann <i>et al.</i> (1992)
pACB1	Recombinant cosmid able to complement <i>E. coli</i> DV21A05	This work
pACYC184	Cm ^R Tet ^R <i>oriP15A</i>	Chang & Cohen (1978)
pAB-17	pACYC184 containing a 5.5 kb insert from cosmid pACB1	This work
pEK0	<i>E. coli</i> - <i>C. glutamicum</i> shuttle vector, Ap ^R Km ^R	Eikmanns <i>et al.</i> (1991a)
pEKB1	pEK0 containing a 3 kb <i>BfrI</i> - <i>PvuI</i> fragment from pAB-17	This work
pEKA2	pEK0 containing a 2.4 kb <i>HpaI</i> - <i>NciI</i> fragment carrying the <i>C. glutamicum aceA</i> gene	Reinscheid <i>et al.</i> (1994)
pUC18	Ap ^R	Vieira & Messing (1982)
pSUP301	Mobilizable vector, <i>oriT</i> , Km ^R	Simon <i>et al.</i> (1983)

* Cm^R, chloramphenicol resistance; Tet^R, tetracycline resistance; Km^R, kanamycin resistance; Ap^R, ampicillin resistance.

† This strain was kindly provided by Barbara Bachmann, *E. coli* Genetic Stock Center, New Haven, USA.

of malate synthase were prepared as described above except that the cells were washed in 50 mM 2-morpholinoethanesulphonic acid (MES) buffer, pH 6.0, and resuspended in 10 ml of the same buffer containing 5 U DNase, 15 µg RNase and 100 µM phenylmethanesulphonyl fluoride (PMSF). All purification steps were carried out at 4 °C. After ultracentrifugation of the crude extract at 183000 g, the supernatant was subjected to FPLC anion-exchange chromatography using a MonoQ HR5/5 column with a 0.1–0.4 M NaCl gradient (flow rate: 1 ml min⁻¹). For the second chromatography the buffer of the partially purified enzyme was changed to 50 mM Tris/HCl, pH 8, by ultrafiltration with an Ultrafree filter cup (Millipore). During the second MonoQ HR5/5 chromatography 50 mM Tris/HCl, pH 8, was used with a 0.2–0.5 M NaCl gradient and a flow rate of 1 ml min⁻¹.

SDS-PAGE, gel filtration and determination of the N-terminus of malate synthase. SDS-PAGE was performed using a Hoefer Studier vertical slab gel instrument (Serva) in 12.5% (w/v) polyacrylamide gels overnight at 8 mA according to the method of Laemmli (1970). Gel filtration was performed by FPLC using a Superdex 200 column (Pharmacia). The buffer consisted of 100 mM Tris/HCl, pH 7.6, 100 mM NaCl and 5 mM MgCl₂. Protein standards were catalase (*M_r* 232000), aldolase (*M_r* 158000), BSA (*M_r* 67000) and ovalbumin (*M_r* 43000). For determination of the malate synthase N-terminus, the purified enzyme was subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane filter and sequenced using an Applied Biosystems 477A sequenator equipped with a Blot cartridge and an on-line HPLC apparatus 120.

RESULTS

Isolation of the *C. glutamicum* malate synthase gene *aceB*

In order to isolate the *aceB* gene from *C. glutamicum* a pHC79-based cosmid gene library from this organism was transformed into the *E. coli* mutant DV21A05. Due to its malate synthase deficiency strain DV21A05 is not able to grow on acetate minimal medium (Vanderwinkel & De Vlieghere, 1968). By testing approximately 1000 transformants for growth on acetate minimal medium three clones were obtained which grew on acetate as carbon source. After isolation of the cosmids pACB1, pACB2 and pACB3 and retransformation into *E. coli* DV21A05, the transformants again grew on acetate minimal medium, suggesting that the cosmids carried the *C. glutamicum aceB* gene.

In order to subclone the *aceB* gene, cosmid pACB1 was partially digested with *Sau3A* and fragments between 3 and 6 kb were ligated into the *Bam*HI site of the *E. coli* vector pACYC184. The ligation mixture was transformed into *E. coli* DV21A05 and chloramphenicol-resistant transformants were again screened for growth on acetate minimal medium. By this procedure nine clones carrying plasmids able to complement the malate synthase deficiency of *E. coli* DV21A05 were obtained. The

Fig. 1. Restriction maps of the chromosomal *C. glutamicum* fragments carrying (a) the malate synthase gene *aceB* and (b) the isocitrate lyase gene *aceA*. The fragment sequenced in the present study is marked by bold letters. The respective open reading frames are indicated by arrows. As, *Asp700*; Bf, *BfrI*; Cl, *Clal*; Hp, *HpaI*; Kp, *KpnI*; Nc, *NcoI*; Ni, *NciI*; Nr, *NruI*; Pv, *PvuI*; Sm, *SmaI*; Sp, *SphI*.

Table 2. Specific activities of malate synthase in crude extracts of *E. coli* and *C. glutamicum* strains

The cells were grown in minimal medium (MM) containing glucose or acetate as carbon source. The values are means $\pm$ standard deviation obtained from at least three independent cultivations by two determinations per experiment. NG, No growth.

Strain	Malate synthase activity [U (mg protein) ⁻¹]	
	MM + glucose	MM + acetate
<i>E. coli</i> DV21A05	< 0.01	NG
<i>E. coli</i> DV21A05 pHC79	< 0.01	NG
<i>E. coli</i> DV21A05(pAC1)	0.35 $\pm$ 0.06	0.30 $\pm$ 0.03
<i>E. coli</i> DV21A05(pAB-17)	0.29 $\pm$ 0.04	0.24 $\pm$ 0.04
<i>E. coli</i> DV21A05(pEKB1)	0.24 $\pm$ 0.02	0.29 $\pm$ 0.04
<i>C. glutamicum</i> WT	0.04 $\pm$ 0.01	2.11 $\pm$ 0.22
<i>C. glutamicum</i> (pEK0)	0.04 $\pm$ 0.01	2.23 $\pm$ 0.13
<i>C. glutamicum</i> (pEKB1)	0.35 $\pm$ 0.04	6.22 $\pm$ 0.40
<i>C. glutamicum</i> ALB1	< 0.01*	NG

* When *C. glutamicum* ALB1 was grown in minimal medium containing glucose plus acetate, malate synthase activity could also not be detected.

restriction map of the 5.5 kb insert from one of the plasmids, pAB-17, is shown in Fig. 1(a).

To localize the *aceB* gene more precisely, defined fragments were isolated out of plasmid pAB-17 and ligated into the *E. coli*-*C. glutamicum* shuttle vector, pEK0. The resulting plasmids were transformed into *E. coli* DV21A05 and tested for complementation ability. By this procedure, plasmid pEKB1 was obtained carrying a 3 kb *BfrI*-*PvuI* fragment which was able to complement the *E. coli* mutant.

Southern hybridization was performed to confirm that the cloned 3 kb *BfrI*-*PvuI* fragment originated from *C. glutamicum*. Chromosomal DNA from *C. glutamicum* wild-type (WT) was digested with *BfrI* and *PvuI*, size-fractionated and transferred onto a nylon membrane. A 0.84 kb *SmaI*-*KpnI* fragment was isolated from pEKB1, labelled and used as a probe. This fragment hybridized specifically to a chromosomal fragment of 3 kb (not

shown) confirming that the isolated *aceB*-complementing fragment originates from *C. glutamicum* and that it corresponds to a fragment within the genome with no detectable structural alteration.

Malate synthase activity in *E. coli* and *C. glutamicum*

The specific malate synthase activities in crude extracts of *E. coli* DV21A05 clones carrying cosmid pAC1, plasmid pAB-17 or pEKB1 were determined after growth on minimal medium containing glucose or acetate. As shown in Table 2 the three strains displayed considerable malate synthase activities [between 0.24 and 0.35 U (mg protein)⁻¹] both after growth on glucose and acetate minimal medium. No malate synthase activity was detected in the host *E. coli* DV21A05 and in *E. coli* DV21A05 carrying cosmid pHC79 without insert (Table 2). These results confirm that the isolated fragments contained the malate synthase gene from *C. glutamicum* and show that it is expressed in *E. coli*.

For homologous expression of the isolated *aceB* gene, plasmid pEKB1 was introduced into *C. glutamicum* WT and the specific malate synthase activities of the host and of the transformant, *C. glutamicum*(pEKB1), were determined (Table 2). After growth on glucose minimal medium the recombinant strain displayed about ninefold higher specific malate synthase activity relative to *C. glutamicum* WT or its derivative carrying the cloning vector pEK0. When acetate minimal medium was used for growth, the specific malate synthase activities of *C. glutamicum* WT and *C. glutamicum*(pEK0) were approximately 50-fold higher than after growth on glucose minimal medium, and that of *C. glutamicum*(pEKB1) was still threefold higher compared to the host (Table 2). These results show that the malate synthase of *C. glutamicum* is tightly regulated by the carbon source and indicate that the isolated *BfrI*-*PvuI* fragment carries the *aceB* gene including the structures necessary for expression and regulation in *C. glutamicum*.

Nucleotide sequence of the *aceB* gene

The DNA sequence of the 3024 bp *BfrI*-*PvuI* fragment was determined from both strands by the dideoxy chain-termination method. The complete nucleotide sequence of the *C. glutamicum* *aceB* gene including 5' and 3' regions and the deduced amino acid sequence of the malate synthase is shown in Fig. 2. Computer analysis revealed

1 CTTAAGTCGTGATTCGCAATGGGCGGTCGCACCAAGATGACGTAATGCATGTCGCTGTTTCGACGTGATGTCATCGGTTTCGGC
91 TGGTGGCGTGGTTCACACATGCTCCATCGGGCATTGGTGGCTCAATCGGTTTGGGTTTTAAGTTTTTGTGGGGGGTGGTCAACCCCTGT
181 TGTGAAGTTTGCMAAGTTCTGGCTTCGCAAGAAAAGTGGCGGGGGAGTTGCTATACGGATGATCATGGGCAATGCTCTGAATGGGA
271 AATCGACGACGCAACGTTCCGTAGTTTCTGGAAGTGTGACCTAGATAAAGTTCGGGTTAGGCGGGGGTAATGACTTACTGAATTTGCG
361 CAACCCCTTTTGTGGTGCAGCTGATCATCTTAGTCTGATCAGTCCGCAACACGATGAAGGTTGAATTCGAAGAAGATGGCACCTA
451 GATTCACAGGTAGTCAGAGTCTTTCTTAAAGAGTTTTCACAAACGCTTAACGGCTAGCCAAACAGAGGATTCGCACTTCTCTGGT
541 TTAGGACAGGTCATCTAAACCCATGCTTTAAAGAGGCTTCCTCAAGCTGACAGGAAGATCTGTCTGCTCAGACTGGCGCAACGCT
RBS MTEQELLSAQTDNA
631 GGAATCGACAGCACCAGCGGTTGACCGCGGGGAATCGAGTTTGCAGAAATCTCTACGACTTGTGAACGAGCGGCTACTCCCTCGC
GTDSTERVTDAGGGMQVAKVLVDVFVTEAVPLR
721 GTGGTGTGGATGGGAAAATTTGGTCCGATTCGCGCCGACCGGGGACCTCCACGCAACGCGGAGCTGCTGTCTGGCGCG
VGVDAEAKFWSGFAAIARDLTPRNRELLARR
811 GATGACCTGCAGATGCTTATCGACGACTACCAACCAACCTCGGCGACCTGACGACGAGGCTGACGAGGATTTCTCTCAAGAAAT
DELMQLIDDYHRRNNSGTIDQEAYEDFLKEI
901 GGATACCTGTGTTGAGGACCGAGAAGTCGAGAAATCCGATCCCAACGCTGATACGAAATTCGACACCGGAGGACTCGAGTGGTT
GYLVEEPEAAAEIERTQNVDTETISSTAGPQLV
991 GTTCCTATCTGAACGACGTTTCGCGTGAACGCTGCCAATGCTCGCTGGGTTCCCTCTACGATGGTGTGACGACCAACCGCACT
VPLNLNARNAANRANRNLVLDALYGTGNNAI
1081 CCAGAATCTGATGGCGTGAAGAAGGCAAGGATCAACCCGCTCCGCGGCGAAGGCTCATGATGGGCTGTGAATCTCTGCACAG
PETIDGAEGKEGKEYMPVRGQKVIENGREFLDS
1171 GTTGCCCATCTGACGGCTGTCGATCCGATGTGTGAGAGATCAACATCAGCATGGAAGCTTGCAGCCCATGTGAGATGATGCTGT
VYPLDGAASHADVEKEYNITDGGKLLAAHIGDSV
1261 TACCGACTGAAGAACCTGGAATCTACCTGGGTTTACCGGAGCTCTTGATCTCAAGAACCTCTGCTGGAACACACGCGCTGCAC
YRLKNRESRYRGTGTGNFLDVEAAILLEINGLH
1351 ATCGAGCTGACATGATCTGTGACCCTAATCGCAAGGCAGACAAAGTGGCTTCAAGACATCGTTTGGAACTTCGATCACCAG
IEIQIDPVPHPPIGKADKTGLKDIVLESATIT
1441 ATCATGCTTCGAAGACTCGGTGACGCTGTGATGCTGAAGCAAGACTTAGTGTCTAAGCTTACGTTTCGAGCTCAACACGGCGAA
IMDFEDSVAADVAAGDCTGLTGSYNWFLGNTGE
1531 CTGAAGAAGAGATGTCGAAGACGAGCATCTCACCCGTGAGCTCAACAGGACCGGCTACATGCGCGCAATGGTACGAGTG
LKEEMNSKGRIFRRELNKDARVYVIGRNGTE
1621 GTTCTGCAGCGCTGCTCCCTGGTTGTGTGTCGCGAAGTGGTCACTTCAAAACCACTTCATTTGATGGCGAGGAGATCTTC
VLHGRSLLFVRNVGHLMQNPSSIJDGEEIF
1711 GAGGACTCATGATGCTGTCTGCACACTTGTGTGGCCATCGAGGAATGTCTCCGCAAGCAAGATGCGCAATTCGCGCAAGGCTCC
EGIMDAVLTVTTCATPCTGATPQIAPQKNMNRNSRKGGS
1801 ATCTACATCGTGAAAGCTAAGCAGCAGGCGCTGAAGAAGTCGCTTACCAACGAGCTTCTGGCGCGGTTGAGGATCTGATGATCTG
IYIYKPKQHGPEEVAFTINELFGRVEDLLDL
1891 CCAGCCCACTTGAAGGTTGGTGTATGATGAGGAGGCTGCACGCTCGTGACCGTGAATGCCACATCAAGGATGCTGTGACCG
PRHTLKTGVNWDEERTRSVMNLDSMEVADR
1981 TTGGCATCTCAACACTGGCTTCTGGACCGACCGGCTGAATACACCTCCATGAAGCAGGCGCATGTGCGCAAGGCTGAT
LAFINTGTGATCTGDEIHTSMEAGAMVRKAD
2071 ATCGACACCGCAGCTGAAGCAGGCTGACGAGCAACCAAGCTGATGACGAGTATCAGCGGTTCTCTGCGAAGCTCATGATCGGT
MQIAPNKKQAYENNNNDVAGAGAQCGGLPQGLPGKAQIG
2161 AAGGCGATGTGGGCGATCTGAACCTATGCAAGAACTGGAGAAGAAGTGGCGGACGACGCAAGCGCCCAACATGATGGTT
KGMWAMTELMHAEMLEKKIGQPREGANTGV
2251 CCTTCAACACTGGTGACGCTGCACGACGACATACCACTGGTTGATGTGTCAAGGTTCAAGACAGATGCGTGTGCGCGCCG
PSPSTGATLHATHATYHYLVLDVFKVQDELRAGAAR
2341 CGCGACGCTCGCGCAATCTCCACATTCCACCGCCCAACACCAATTTGCTGAGGAAGAGAGGAAGGAAATGGACACAC
RDSLRLNLTITPAPTINWSEEEKKEEMDN
2431 TGCCAGTCACTCTCGGATAGTTTGTGCGGTGGTGTGACACGGTTTGGTTGCTCAAGGTTTCAGACATCCATGACATGCACTCATG
CQSIILTYGVVRVWVHGVGVCSSKVPDIIHDI
2521 GAGACGCGGACGCTGCGATTTCTTCGAGGATGTGGCCCACTGATCGGCGCATGTTGTCTCGAAGGACAGGCTTTGGAGTCA
EDRATLRISSQMLANWHRHDVVSKEQVLES
2611 CTGGAACGATGAGCAGTGGTCTGCGACAGCAAGTCCGCGGACGAGGCTACCGCATATGGCGCGCACTACGACGCTCTCTCGCC
LERHAWVVDGKKNNAAGDEAYRDHAPNYDASLA
2701 TTCAGCGCGCTAAGGACTGATTTTGAAGGACCAAGTCCCATCGGGCTACACCGGACCATCTTGCACGACGCGCGCGAGTGT
FQAACKDLIFEGTKSPSGYTEPILHARRREF
2791 AAGCAAAAACATGACGCTTTTCAGCCTTACTGTGATCCCAACGCGTACGACTGCCCGGAGCACCCTCACTCTTTTGTGTCA
KAKN
2881 GCACCCAAAGGCGCGGTTCAACACACCAAGTCGGCGATTACCTTCCGCAATTCGCGACGGTGGAGCGCGCATTTCCGCTGGA
2971 TTCCACACACAGTGGAAATCATGACCTGCGCCCTCAATGGTGATGATGCGATG

Fig. 2. Nucleotide sequence of the 3024 bp *BfrI*-*PvuII* fragment carrying the *aceB* gene from *C. glutamicum*. The predicted amino acid sequence of the malate synthase is shown below the nucleotide sequence. A putative ribosome-binding site is indicated as RBS and a potential terminator structure is marked by arrows.

one large open reading frame (ORF) extending from nucleotide 586 to 2802 and exhibiting a codon usage corresponding to highly expressed corynebacterial genes (Eikmanns, 1992). The predicted translational initiation

codon at nucleotide 586 is preceded by a typical ribosomal binding site. Downstream of the ORF, at positions 2910–2962 in Fig. 2, a potential rho-independent termination structure was found. According to the rules of Tinoco *et al.* (1973) this palindromic structure should be capable of forming a stem-loop with a ΔG (25 °C) of $-41 \text{ kcal mol}^{-1}$ ($-172.2 \text{ kJ mol}^{-1}$). The ORF encodes a polypeptide of 739 amino acids with an M_r of 82362. Since no other ORF of significant size was found in either orientation on the *BfrI*–*PvuI* fragment, it was evident that the predicted polypeptide represents the malate synthase from *C. glutamicum* and the ORF was therefore named *aceB*.

Comparison of the deduced malate synthase amino acid sequence from *C. glutamicum* with those of other organisms

Comparison of the deduced amino acid sequence of the *C. glutamicum* malate synthase with sequences of enzymes from other organisms revealed only weak similarity. Whereas the predicted *C. glutamicum* *aceB* gene product consists of 739 amino acids, malate synthases of other organisms consist of between 533 and 568 amino acids (for overviews, see Hikida *et al.*, 1991 and Fernandez *et al.*, 1993), suggesting that the *C. glutamicum* enzyme is larger by at least 170 amino acids. Within the N-terminal 200 amino acids of the *C. glutamicum* malate synthase no similarity at all to other malate synthases could be detected. However, since residues important for the catalytic mechanism may be found among conserved amino acids, the deduced amino acid sequence of the *C. glutamicum* malate synthase starting at amino acid 160 was aligned with the malate synthase sequences of *E. coli* (Byrne *et al.*, 1988), *S. cerevisiae* (Hartig *et al.*, 1992; Fernandez *et al.*, 1993), the ascomycete *N. crassa* (Sandemann *et al.*, 1991) and of the plant *Brassica napus* (Comai *et al.*, 1989) (Fig. 3). The aligned region of the *C. glutamicum* enzyme shows less than 15% identity to any of the other malate synthases, whereas these show overall identities of between 42 and 49% among each other and possess several highly conserved regions (underlined in Fig. 3). It is striking that the few amino acids identical in all five aligned sequences are primarily located within these highly conserved regions.

The *aceB* gene is located in close proximity to the *aceA* gene

Recently, we isolated and sequenced a 2.43 kb *HpaI*-*NciI* fragment (Fig. 1b) carrying the *C. glutamicum aceA* gene encoding isocitrate lyase (Reinscheid *et al.*, 1994). Comparison of the nucleotide sequence of this *HpaI*-*NciI* fragment with that of the *aceB*-carrying *BfrI*-*PvuI* fragment revealed that both fragments share a common region, i.e. the first 504 nucleotides of the *BfrI*-*PvuI* fragment in Fig. 2 corresponded to the inverted nucleotide sequence in front of the *aceA* gene. From these data it could be predicted that in *C. glutamicum* the *aceB* gene is clustered together with *aceA*, that both genes are separated by 597 bp and that they are oriented in opposite directions.

CGMS	...GTNAIPETDGAEGKEYNPVRGQKVIEWGREFLDSVPLDGASHADVEKYNITDGKLAHIGDSVYRLKNRESYRGFTGNFLDPEAILLE	250
ECMS	MTEQAT-----TTDELAFTRPYGEQEQILTAEAVEFLTELVTHTPQR--NKLLAARIQQQDIDNGTLP-DFISETASIRD	75
SCMS	M---VKVSL--DNVKLLVDVDKEPFFKPSSTTVGDILTDALEFIVLLHRTFNKKR--KQLLENRQVQKKLDSGSYHLDLFPETANIRN	83
NCMS	M---ASVE-----TLLQGVTISG---PIEEHQKILTPQALSFVALLHRSFNQTR--KNLLERRHVRQAEIDRGVLP-DFLPETKHIRE	75
BNMS	MELETSVY--RPNVAVYDSPDGEVVRGRYDQVFAKILTRDALGFVAELQREFRGHV--RYAMECRREVKKRYNSGAVP-GFDPSTKFIIRD	85
	SS SS S *	
CGMS	TNGLHIELQIDPVHPIGKADKTGLKDIVLESAITTIMDFEDSVAAVAEDDKTLGYSNWFGLNTGELKEEMSKNGRIFTRRELNKDRVYIGR	340
ECMS	A-DWK-IRGIPADLEDRRVEITGPVERKM-----VINALNANVKVFMADFEDSLAPDWNKVIDGQINLRDAVNGTISYTNEA-GKIYQLK	157
SCMS	DPTWQ-GPILAPGLINRSTEITGPPLRNM-----LINALNAPVNTYMTDFEDSASPTWNNMVYQVNLVYDAIRNQIDFDTPR--KSYKLN	165
NCMS	NPTWKGAAPAAPPLVDRRVENTGPTDRKM-----VVNALNSDVYTYMADFEDSSAPTWANMVNGQVNLVYDAIRNQIDFKQGP--KEYKL-	157
BNMS	G-EWV-CASVPPAVADRRVEITGPVERKM-----IINALNSGAKVFMADFEDALSPSWENLMRGQVNLKDAVDGSITFNDKARNKVYKLN	168
	S ** S S S * S * S *	
CGMS	NGTELVLHGRSLLFVRNVGHLMQNPSILIDGEEIFEGIMDAVLTTVCAIPGIAPQNKMRNSRKGSIIYVKPKQHGPPEEVAFTNELFGRVE	430
ECMS	PN---P---AVLICRVRGLHLEKHTVWRGEAIPGSLDFALYFFHNYQAL-----LAKSGPYFYLPKTSQWQEAANWSEVFSYAE	233
SCMS	GN---V-ANLPTIIVRPRGWHMVEKHLVDDEPISASIFDFGLYFYHNAKEL-----IKLGKGPYFYLPKMEHLEAKLWNDVFCVAQ	244
NCMS	RTDRTL---PTLIVRPRGWHLEEKHVTIDGEPVSGSLDFGLYFFHNAKEL-----VQRGFGPYFYPPKMESHLEARLWDAFNLAQ	236
BNMS	DQ---V---AKLFVRPRGWHLEPAHILIDGEPATGCLVDGLYFFHNYAKF----RQTQSGSGFGPFYLPKMEHSREAKIWNVSFERAE	247
	S * * S S * S * * S ** *	
CGMS	DLDDLPRHTLVKGVMDDEERTSVNLDASIMEVADRLAFINTG---FLDRTGDEIHTSMEAGAMVRKADMQTAPWKQAYENNNVDAGIQRG	517
ECMS	DRFNLPRGTIKATLLIETLPVAFQMDIELHALRDHIVGLNCRWDYIFSYIKTLKNYPDRVLPDRQAVTMDKPFNLAYSRLLIKTHCKRG	323
SCMS	DYIGIPRGRIRATVLIETLPAAFQMEIIYQLRQHSSGLNCRWDYIFSTIKRLRNDPNHILPNRNQVTMTSPFMDAYVKRLINTCHRRG	334
NCMS	DYVGIPLSTIRGTVLIETITAAFQMEIIFELRNHTSGLNCRWDYIFPFKEVRRFPNFVLPDRSDVTMTVPFMEAYVKLLIKTLHRLV	326
BNMS	KMAGIERGSIRATVLIETLPVAFQMEIILYELRDHSVGLNCRWDYIFSYVKTFAHPDRLLPDRVLVGMGQHFMRSYDILLIRTHCKRG	337
	S SSS SS * S S S S S * * SS S S *	
CGMS	LPGKAQIGKGMWAMTELMAEMLEKKIQGPREGANTAWVPSPTGATLHATHYHL-VDVFKVQDELRAAGRRDSLRLNLTPTAPNTNWSEE	606
ECMS	AFAMGGMAAFIPSKDEEHNQV-LN-KVKADKSLEANNHGDGTWIAHPGLADTAMAVFNDILGSRKNQLEVMREQDAPITADQLLAPCDG	411
SCMS	VHAMGGMAAQIPKDDPAANEKAMTKVRN-DKIRELTNGHDSWVAHPALAPICNEVFINMG-TPNQIYFIPENVVTAAN-LLETIKIPNG	421
NCMS	VHAMG-MAAQIPKDDKAANDKAMEGVVRADKLREARAGHDGTWVAHPALASIALEVFNKHMPNPNQLFNRREDVKIGQQDLLNMVNP-G	414
BNMS	VHAMGGMAAQIPKDDPKANEMALD-LVKKDKLREVRAGHDGTWAAHPGLIPICMDAFSHMGNNPNQIKSMKRDDASAITEDLLQIPRG	426
	S S S *	
CGMS	EKKEE-MDNNCQSILGYVVRVVEHGVGCSKVPDIHDIDLMEADRATLRISQMLANWIRHVVSKQVLESLEMAVVVD-----KQ	686
ECMS	ERTEEGMRANIRVAVQYIEAWI-SGNGCVP-----IYGLMEDAATAEISRTSIWQIHHQKTLN---GKPVTKALFRQMLGEEMKVIAS	492
SCMS	EITTEGIVQNLDIGLQYMEAWL-RGSGCVP-----INNLMEDAATAEVSRCQLYQVVKHGVTLKDTG---EKVTPTEKILKEQVERLSK	503
NCMS	SSTEDGIRKNLNTGLGYTEPIW-RGVGCV-----IKHPQEDAATAEVSRSQVWVKKHRTTAEKG---HVDKRYPLKLLKEADQRLAK	496
BNMS	VRTLEGLRLNTRVGIIQYLAWL-TGSGSVP-----LYNLMEDAATAEISRVQNWQWIRYGVLDGDLGVRVSKELFGRVVEEEMERIEK	510
	S S * S * * S * * ** * S * * SS	
CGMS	-NAGDEAYRDMAPNYDASLAFQAADLIFEGTKSPSGYTEPILHARRREFKAKN	739
ECMS	-ELGEERFSQGRFDDAARLMEQITTSDELIDFLTLPGYRLLA-----	533
SCMS	ASPLGDKNKFAL---AAKYFLPEIRGEKFSEFLTLLYDEIVSTKATPTDLSKL	554
NCMS	-APQGNKFNLA---AQYFASQVTGE-DYADFLTCLLYNEITSAGNSLPASKL-	543
BNMS	-EVGKDKFKRGMKEACKMFTKQCTAAELDDFLTAVYDHIIVAHYP-INASRL-	561
	S *	

Fig. 3. Alignment of the predicted malate synthase sequences from *C. glutamicum* (CGMS), *E. coli* (ECMS) (Byrne et al., 1988), *S. cerevisiae* (SCMS) (Hartig et al., 1992; Fernandez et al., 1993), *N. crassa* (NCMS) (Sandemann et al., 1991), and *B. napus* (BNMS) (Comai et al., 1989). Identical and similar amino acids are marked by * and s, respectively. Regions of high identity between the enzymes from *E. coli*, *S. cerevisiae*, *N. crassa* and *B. napus* are underlined.

To ensure that both genes are clustered on the *C. glutamicum* chromosome, Southern hybridization was performed. An *aceB*-specific 0.84 kb *Sma*I-*Kpn*I fragment and an *aceA*-specific 0.92 kb *Asp*700-*Bfr*I fragment (see Fig. 1a and b) were used as probes. Hybridization of *Asp*700-digested chromosomal *C. glutamicum* WT DNA

to the *aceA*-specific and the *aceB*-specific probes resulted in both cases in a signal at 4.9 kb (not shown). This result was expected from the combined restriction maps of the *aceA*- and *aceB*-carrying fragments (see Fig. 1). Thus, the *aceA* and *aceB* genes of *C. glutamicum* are in fact clustered and oriented in antiparallel direction as shown in Fig. 1.

Table 3. Purification of malate synthase from crude extracts of *C. glutamicum*(pEKB1) after growth in acetate minimal medium

Fraction	Vol. (ml)	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Recovery of activity (%)	Purification factor (-fold)
Crude extract*	2.3	66.7	415.4	6.2	100	1.0
Ultracentrifugation	18.0	30.8	408.2	13.3	98	2.1
MonoQ, pH 6.0	3.0	3.9	109.2	27.8	26	4.5
MonoQ, pH 8.0	2.0	3.5	101.4	28.6	24	4.6

* Prepared from 1 g cells (wet weight).

Inactivation of the chromosomal *aceB* gene in *C. glutamicum*

To investigate whether *C. glutamicum* requires a functional *aceB* gene for growth, the chromosomal *aceB* gene of *C. glutamicum* WT was inactivated by gene disruption. The resulting strain, *C. glutamicum* ALB1, was then tested for malate synthase activity and for growth on different media. The specific malate synthase activity was determined in crude extracts of *C. glutamicum* ALB1 after growth in minimal medium containing glucose or glucose plus acetate as carbon sources. As shown in Table 2 strain ALB1 was devoid of detectable malate synthase activity. Growth of *C. glutamicum* ALB1 on minimal medium containing glucose as carbon source was identical to that of the parental *C. glutamicum* WT. However, in contrast to the WT strain, *C. glutamicum* ALB1 was not able to grow on acetate as sole carbon source. When a mixture of acetate and glucose was given as carbon source, growth of *C. glutamicum* ALB1 was significantly impaired in comparison to the parental strain (doubling times of 180 min and 100 min, respectively). It can be speculated that due to malate synthase deficiency the mutant accumulates glyoxylate internally and this might be detrimental for the cells. These results show that the *aceB* gene in *C. glutamicum* ALB1 is inactivated and that *C. glutamicum* requires a functional *aceB* gene for growth on acetate. It can therefore be suggested that in *C. glutamicum* only one malate synthase exists.

Purification and characterization of malate synthase

The analysis of the *aceB* gene from *C. glutamicum* and the alignment studies suggested that malate synthase from this organism differs significantly from hitherto known malate synthases both in size and primary structure. In order to obtain information about the biochemical characteristics of the *C. glutamicum* enzyme, the malate synthase was purified and biochemically analysed. The *aceB*-overexpressing strain *C. glutamicum*(pEKB1) was grown on acetate minimal medium and used for purification of the malate synthase. Table 3 summarizes the purification procedure and gives representative data of the preparation. The enzyme was purified 4.6-fold to apparent homogeneity (Fig. 4), indicating that malate synthase represents about 20% of the cytoplasmic protein

Fig. 4. SDS-PAGE of crude extract from *C. glutamicum*(pEKB1) after growth in acetate minimal medium (lane 1) and SDS-PAGE of purified malate synthase (lane 2). *M*, standards (lane M): α_2 -macroglobulin (170 kDa), β -galactosidase (116 kDa), fructose-6-phosphate kinase (85 kDa), glutamate dehydrogenase (56 kDa), aldolase (39 kDa), triosephosphate isomerase (27 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa).

fraction within *C. glutamicum*(pEKB1). This value is in good agreement with the relative amount of malate synthase that can be seen after SDS-PAGE of crude extracts from acetate-grown *C. glutamicum*(pEKB1) (Fig. 4). The SDS-PAGE analysis of the purified enzyme revealed a single protein band with an M_r of about 90 000

(Fig. 4), which corresponds reasonably well to the M_r predicted from the *aceB* sequence (82362, see above). By gel filtration the native M_r of malate synthase was determined to be about 80000. These results suggest that the malate synthase from *C. glutamicum* is a monomer in its native form and confirm that the *C. glutamicum* enzyme is in fact significantly larger than the hitherto known malate synthase monomers from other organisms (for overviews, see Cioni *et al.*, 1981; Hikida *et al.*, 1991; Fernandez *et al.*, 1993).

In order to confirm the predicted translational initiation site, the N-terminus of purified malate synthase was sequenced. The amino acid sequence obtained was T-E-Q-E-L-L-S-A-Q-T and thus corresponds to the sequence deduced from the nucleotide sequence of the *aceB* gene. The missing methionine in the purified malate synthase suggests that it is removed by processing.

The purified *C. glutamicum* malate synthase remained stable at 4 °C for more than 3 weeks without loss of activity. The enzyme showed a pH optimum of 7.6 in 50 mM Tris/HCl buffer, a temperature optimum of 43 °C and exhibited an absolute requirement for divalent cations. Maximum activity was observed in the presence of 40 mM Mg^{2+} . Co^{2+} or Mn^{2+} could partially replace Mg^{2+} resulting in 25% and 15%, respectively, of the maximal activity. The K_m values determined for acetyl CoA and glyoxylate were 12 μ M and 30 μ M, respectively, which is in the same range as previously reported for malate synthases from other organisms (Cioni *et al.*, 1981).

To analyse whether malate synthase is regulated on the enzymic level, its inhibition by several effectors was studied. Oxalate and glycolate, both structural analogues of glyoxylate, were found to be competitive inhibitors with regard to glyoxylate. The inhibition constants of oxalate and glycolate were 0.27 mM and 0.44 mM, respectively. ATP turned out to be a competitive inhibitor versus acetyl CoA showing an inhibition constant (K_i) of 4.3 mM. No effect on enzyme activity was observed with fructose-1,6-bisphosphate, pyruvate, phosphoenolpyruvate, acetyl phosphate or with any intermediate of the citric acid cycle. From these results we assume that in *C. glutamicum* the inhibition or activation of the malate synthase plays only a minor role in controlling the carbon flux in the glyoxylate cycle.

DISCUSSION

The data reported above describe the isolation and characterization of the *C. glutamicum* malate synthase gene *aceB*. To our knowledge, this is the first *aceB* gene isolated from a Gram-positive organism, and besides that of *E. coli* it represents the only malate synthase gene sequenced from a prokaryote. By sequence analysis and hybridization studies, evidence was provided that the *aceB* gene in *C. glutamicum* is clustered together with the isocitrate lyase gene *aceA*, which has been isolated and sequenced previously (Reinscheid *et al.*, 1994). Both genes are transcribed divergently and thus the genomic organization of *aceB* and *aceA* is different from that in *E. coli*. In this organism, and probably also in *Salmonella*

typhimurium, the two genes constitute an operon together with the isocitrate dehydrogenase-kinase/phosphatase gene *aceK* in the order *aceB-aceA-aceK* (Brice & Kornberg, 1968; Maloy & Nunn, 1982; Wilson & Maloy, 1987; Chung *et al.*, 1988; Cortay *et al.*, 1989). The expression of this *ace*-operon is negatively controlled by the *iclR* and *fadR* gene products in a trans-dominant manner (Maloy & Nunn, 1982), and due to this regulation both malate synthase A and isocitrate lyase are only formed when acetate or long-chain fatty acids are the sole carbon sources. In *C. glutamicum*, the formation of both enzymes is also drastically increased during growth on medium containing acetate (Reinscheid *et al.*, 1994; this work). However, the antiparallel genomic organization of the *aceB* and *aceA* genes in *C. glutamicum* predict that in contrast to *E. coli* both genes are expressed independently by their own promoters. This poses the interesting question about the co-ordinated regulation of the *C. glutamicum* *aceB* and *aceA* gene expression. It is likely that the two genes are controlled by the same regulatory mechanism, perhaps even at the same site on the DNA. Both genes may also be regulated differentially in their expression allowing adaptation to growth under conditions in which only one of the two enzymes is necessary, e.g. growth on glycolate, glyoxylate or allantoin (Vanderwinkel & De Vlieghere, 1968; Hartig *et al.*, 1992). However, *C. glutamicum* is not able to grow on these compounds (unpublished results) and at present our data do not allow any conclusion about the regulation of *aceB* and *aceA* expression in *C. glutamicum*.

Biochemical analysis of the *C. glutamicum* malate synthase indicates that the native enzyme is a monomer, that it requires divalent cations, that it shows high affinity to its substrates and that it is effectively inhibited by oxalate, glycolate and ATP. With respect to these features the *C. glutamicum* enzyme is very similar to malate synthases purified from other bacteria (Dixon *et al.*, 1960; Chell & Sundaram, 1975, 1978) and, with the exception of its monomeric nature, also to those from eukaryotes (Cioni *et al.*, 1981; Okada *et al.*, 1986; Fukawa *et al.*, 1987). Despite these functional similarities, the *C. glutamicum* malate synthase is remarkably different in its size and primary structure. Compared to all other known malate synthases the enzyme from *C. glutamicum* has an extra N-terminal stretch consisting of about 170 amino acids, long enough to form a distinct domain. However, a database search with this N-terminal sequence revealed no similarity to any known sequence in the SWISSPROT database. Since the malate synthases from other organisms lack this N-terminal stretch, it is unlikely that it is essential for catalytic activity or known regulatory features. Thus, the extra N-terminus may have some role in structural stability or in the specificity of an as yet unidentified effector of the enzyme.

In addition to the N-terminal extension of malate synthase from *C. glutamicum* its primary structure showed only weak similarity to that of other known malate synthases. One possible explanation is that the *C. glutamicum* enzyme shares a common ancestor with the malate synthases from the other organisms but, due to the N-terminal extension

of the *C. glutamicum* enzyme, its C-terminal part had to undergo significant changes in primary structure to remain functional. It might also be that the *C. glutamicum* enzyme is not homologous to the malate synthases from the other organisms. However, we favour the former hypothesis since our alignment shows that several of the identical amino acids within the aligned sequences are located in the highly conserved regions of the malate synthases from other organisms. It is enticing to speculate that these residues are most important for the catalytic and/or regulatory properties of malate synthases. To our knowledge, no specific amino acids involved in the catalytic or regulatory mechanism have yet been identified experimentally and, due to the high identity of all hitherto known malate synthases, it was up to now impossible to propose active site residues by alignment studies. In this context it is worth mentioning that malate synthase and citrate synthase catalyse similar reactions, i.e. a condensation of acetyl CoA and a 2-oxoacid. Citrate synthase from pig-heart has been crystallized and the three-dimensional structure elucidated (reviewed by Wiegand & Remington, 1986). By X-ray analysis, as well as by site-directed mutagenesis, histidine₂₇₄ and aspartate₃₇₅ were identified as involved in the reaction mechanism (Alter *et al.*, 1990; Wiegand & Remington, 1986). It is striking that in the C-terminal region of all malate synthases, including that of the *C. glutamicum* enzyme, a highly conserved histidine and a highly conserved aspartate residue separated by about 80 amino acids could be identified (H₅₆₄ and D₆₄₇ in the *C. glutamicum* enzyme). However, further experiments are needed to conclusively identify the amino acids important for the catalytic and the regulatory mechanisms of malate synthase enzymes.

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REFERENCES

- Akashi, K., Shibai, H. & Hirose, Y. (1979). Comparison between acetic acid and glucose as a substrate in threonine fermentation. *Agric Biol Chem* **43**, 1563–1566.
- Alter, G. M., Casazza, J. P., Zhi, W., Nemeth, P., Srere, P. A. & Evans, C. T. (1990). Mutation of essential catalytic residues in pig citrate synthase. *Biochemistry* **29**, 7557–7563.
- Armitt, S., McCullough, W. & Roberts, W. (1976). Analysis of acetate non-utilizing (*acu*) mutants in *Aspergillus nidulans*. *J Gen Microbiol* **92**, 263–282.
- Birnboim, H. C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol* **100**, 243–255.
- Börmann, E. R., Eikmanns, B. J. & Sahm, H. (1992). Molecular analysis of the *Corynebacterium glutamicum* *gdb* gene encoding glutamate dehydrogenase. *Mol Microbiol* **6**, 317–326.
- Brice, C. B. & Kornberg, H. L. (1968). Genetic control of isocitrate lyase activity in *Escherichia coli*. *J Bacteriol* **96**, 2185–2186.
- Byrne, C., Stokes, H. W. & Ward, K. A. (1988). Nucleotide sequence of the *aceB* gene encoding malate synthase A in *Escherichia coli*. *Nucleic Acids Res* **16**, 9342.
- Chang, A. C. Y. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic plasmid. *J Bacteriol* **134**, 1141–1156.
- Chell, R. M. & Sundaram, T. K. (1975). Isolation and characterization of isocitrate lyase and malate synthase from *Bacillus stearothermophilus*. *Biochem Soc Trans* **3**, 303–306.
- Chell, R. M. & Sundaram, T. K. (1978). Structural basis of the thermostability of monomeric malate synthase from a thermophilic *Bacillus*. *J Bacteriol* **135**, 334–341.
- Chung, T., Klumpp, D. J. & LaPorte, D. C. (1988). Glyoxylate bypass operon of *Escherichia coli*: cloning and determination of the functional map. *J Bacteriol* **170**, 386–392.
- Cioni, M., Pinzauti, G. & Vanni, P. (1981). Comparative biochemistry of the glyoxylate cycle. *Comp Biochem Physiol* **70B**, 1–26.
- Comai, L., Baden, C. S. & Harada, J. J. (1989). Deduced sequence of a malate synthase polypeptide encoded by a subclass of the gene family. *J Biol Chem* **264**, 2778–2782.
- Cortay, J. C., Bleicher, F., Duclos, B., Cenatiempo, Y., Gautier, C., Prato, J. L. & Cozzzone, A. J. (1989). Utilization of acetate in *Escherichia coli*: structural organization and differential expression of the *ace* operon. *Biochimie* **71**, 1043–1049.
- Dixon, G. H. & Kornberg, H. L. (1959). Assay methods for key enzymes of the glyoxylate cycle. *Biochem J* **72**, 3P.
- Dixon, G. H., Kornberg, H. L. & Lund, P. (1960). Purification and properties of malate synthetase. *Biochim Biophys Acta* **41**, 217–233.
- Eikmanns, B. J. (1992). Identification, sequence analysis, and expression of a *Corynebacterium glutamicum* gene cluster encoding the three glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and triosephosphate isomerase. *J Bacteriol* **174**, 6076–6086.
- Eikmanns, B. J., Kleinertz, E., Liebl, W. & Sahm, H. (1991a). A family of *Corynebacterium glutamicum*/*Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene* **102**, 93–98.
- Eikmanns, B. J., Metzger, M., Reinscheid, D. J. & Sahm, H. (1991b). Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains. *Appl Microbiol Biotechnol* **34**, 617–622.
- Falmagne, P. & Wiame, J.-M. (1973). Purification et caractérisation partielle des deux malate synthases d'*Escherichia coli*. *Eur J Biochem* **37**, 415–424.
- Fernandez, E., Fernandez, M. & Rodicio, R. (1993). Two structural genes are encoding malate synthase isoenzymes in *Saccharomyces cerevisiae*. *FEBS Lett* **320**, 271–275.
- Fukawa, H., Ejiri, S. & Katsumata, T. (1987). Purification and some properties of malate synthase from the pollen of *Pinus densiflora* Sieb. et Zucc. *Agric Biol Chem* **51**, 1553–1560.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). Determination of serum proteins by means of the Biuret reaction. *J Biol Chem* **177**, 751–766.
- Graham, I. A., Smith, L. M., Brown, J. W. S., Leaver, C. J. & Smith, S. M. (1989). The malate synthase gene of cucumber. *Plant Mol Biol* **13**, 673–684.
- Hartig, M., Simon, M. M., Schuster, T., Daugherty, J. R., Yoo, H. S. & Cooper, T. G. (1992). Differentially regulated malate synthase genes participate in carbon and nitrogen metabolism of *Saccharomyces cerevisiae*. *Nucleic Acids Res* **20**, 5677–5686.
- Hikida, M., Atomi, H., Fukuda, Y., Aoki, A., Hishida, T., Teranishi, Y., Ueda, M. & Tanaka, A. (1991). Presence of two transcribed malate synthase genes in an *n*-alkane-utilizing yeast, *Candida tropicalis*. *J Biochem* **110**, 909–914.

- Hohn, B. & Collins, J. (1980). A small cosmid for efficient cloning of large DNA fragments. *Gene* **11**, 291–298.
- Kinoshita, S. (1985). Amino acids. In *Biology of Industrial Organisms*, pp. 115–142. Edited by A. L. Demain & N. A. Solomon. London: Benjamin/Cummings Publishing.
- Kinoshita, S. & Tanaka, K. (1972). Glutamic acid. In *The Microbial Production of Amino Acids*, pp. 263–324. Edited by K. Yamada. New York: John Wiley & Sons.
- Kornberg, H. L. (1966). The role and control of the glyoxylate cycle in *Escherichia coli*. *Biochem J* **99**, 1–11.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- LaPorte, D. C., Thorsness, P. E. & Koshland, D. E., Jr (1985). Compensatory phosphorylation of isocitrate dehydrogenase, a mechanism for adaptation to the intracellular environment. *J Biol Chem* **260**, 10563–10568.
- Liebl, W. (1991). The genus *Corynebacterium* – nonmedical. In *The Prokaryotes*, vol. II, pp. 1157–1171. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer Verlag.
- Liebl, W., Bayerl, A., Schein, B., Stillner, U. & Schleifer, K. H. (1989). High efficiency electroporation of intact *Corynebacterium glutamicum* cells. *FEMS Microbiol Lett* **65**, 299–304.
- Maloy, S. R. & Nunn, W. D. (1982). Genetic regulation of the glyoxylate shunt in *Escherichia coli* K12. *J Bacteriol* **149**, 173–180.
- Okada, H., Ueda, M. & Tanaka, A. (1986). Purification of peroxisomal malate synthase from alkane-grown *Candida tropicalis* and some properties of the purified enzyme. *Arch Microbiol* **144**, 137–144.
- Reinscheid, D., Eikmanns, B. J. & Sahm, H. (1994). Characterization of the isocitrate lyase gene from *Corynebacterium glutamicum* and biochemical analysis of the enzyme. *J Bacteriol* **176**, 3474–3483.
- Sambrook, J., Fritsch, E. F. & Maniatis, J. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Sandemann, R. A. & Hynes, M. J. (1989). Isolation of the *facA* (acetyl-coenzyme A synthetase) and *acuE* (malate synthase) genes of *Aspergillus nidulans*. *Mol & Gen Genet* **218**, 87–92.
- Sandemann, R. A., Hynes, M. J., Fincham, J. R. S. & Connerton, I. F. (1991). Molecular organisation of the malate synthase genes of *Aspergillus nidulans* and *Neurospora crassa*. *Mol & Gen Genet* **228**, 445–452.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A. H. & Pühler, A. (1990). High frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. *J Bacteriol* **172**, 1663–1666.
- Schwarzer, A. & Pühler, A. (1991). Manipulation of *Corynebacterium glutamicum* by gene disruption and replacement. *Bio/Technology* **9**, 84–87.
- Segel, I. H. (1975). *Enzyme Kinetics*. New York: Wiley Interscience.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* **1**, 784–791.
- Thomas, G. H., Connerton, I. F. & Fincham, J. R. S. (1988). Molecular cloning, identification and transcriptional analysis of genes involved in acetate utilization in *Neurospora crassa*. *Mol Microbiol* **2**, 599–606.
- Tinoco, I., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M. & Gralla, J. (1973). Improved estimation of secondary structure in ribonucleic acid. *Nature New Biol* **246**, 40–41.
- Vanderwinkel, E. & De Vlieghere, M. (1968). Physiologie et genetique de l'isocitritase et des malate synthases chez *Escherichia coli*. *Eur J Biochem* **5**, 81–90.
- Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- Wiegand, G. & Remington, S. J. (1986). Citrate synthesis: structure, control, and mechanism. *Annu Rev Biophys Biophys Chem* **15**, 97–117.
- Wilson, R. B. & Maloy, S. R. (1987). Isolation and characterization of *Salmonella typhimurium* glyoxylate shunt mutants. *J Bacteriol* **169**, 3029–3034.

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