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

Dieter J. Reinscheid, Bernhard J. Eikmanns and Hermann Sahm

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, of 82362. 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, of about 80000. 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 μM and 12 μM 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 ≈ 60000) whereas the eukaryotic malate synthases are homo-multimers with subunit sizes corresponding to the prokaryotic enzymes. Besides biochemical characterization of the enzyme, genes coding for malate synthase have been
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; Armit 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 (Kinoshta, 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 Kinoshta & 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 glyoxyxate 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* (DY21A05) 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 Smal–KpnI fragment isolated from plasmid pEKBI and the 0.92 kb Asp700–BfI fragment isolated from plasmid pEKA2 were labelled with digoxigenin-DUTP and used as probes for sequencing. The 1.61 kb BfI–KpnI fragment and the 1.79 kb HindIII–PstI fragment from plasmid pAB-17 were blunt-ended and ligated into plasmid pUC18. From the resulting plasmids progressive uni directional 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 as described by Schwarzer & Pühler (1991). An *aceB* internal 0.42 kb *SalI–KpnI* fragment was blunt-ended and ligated into the *SalI* site of the mobilizable *E. coli* vector pSUP901, 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 Erlemeyer 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 microsonification system (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 plots of Lineweaver–Burk slopes versus inhibitor concentrations (Segel, 1975).

**Purification of malate synthase.** Crude extracts for purification
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli DV21A05†</td>
<td>lacZ43, relA1, (speC-glc)363, spoT1, ppc-2, thi-1, aceB, Hfr</td>
<td>Vanderwinkel &amp; De Vlieghere (1968)</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>Mobilizing donor strain</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>C. glutamicum WT</td>
<td>WT strain ATCC 13032</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td><strong>Cosmids/plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHCT7</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Hohn &amp; Collins (1980)</td>
</tr>
<tr>
<td>pHCT79-based gene library</td>
<td>C. glutamicum chromosomal DNA cloned in cosmid pHCT79</td>
<td>Börnemann et al. (1992)</td>
</tr>
<tr>
<td>pACB1</td>
<td>Recombinant cosmid able to complement E. coli DV21A05</td>
<td>This work</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; Tet&lt;sup&gt;R&lt;/sup&gt; oriP15A</td>
<td>Chang &amp; Cohen (1978)</td>
</tr>
<tr>
<td>pAB-17</td>
<td>pACYC184 containing a 5.5 kb insert from cosmid pACB1</td>
<td>This work</td>
</tr>
<tr>
<td>pEK0</td>
<td>E. coli-C. glutamicum shuttle vector, Ap&lt;sup&gt;R&lt;/sup&gt; Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Eikmanns et al. (1991a)</td>
</tr>
<tr>
<td>pEK0B1</td>
<td>pEK0 containing a 3.5 kb BfrI-PstI fragment from pAB-17</td>
<td>This work</td>
</tr>
<tr>
<td>pEK0A2</td>
<td>pEK0 containing a 2.4 kb HpaI-Nol fragment carrying the C. glutamicum aceA gene</td>
<td>Reinscheid et al. (1994)</td>
</tr>
<tr>
<td>pSUP301</td>
<td>Mobilizable vector, oriT, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Simon et al. (1983)</td>
</tr>
</tbody>
</table>

*Cm<sup>R</sup>, chloramphenicol resistance; Tet<sup>R</sup>, tetracycline resistance; Km<sup>R</sup>, kanamycin resistance; Ap<sup>R</sup>, 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-morpholinoethanesulfonic 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<sup>-1</sup>). For the second chromatography the buffer of the partially purified enzyme was changed to 50 mM Tris/HCl, pH 8, by ultrafiltration with a 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<sup>-1</sup>.

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 MgCl<sub>2</sub>. Protein standards were catalase (M<sub>r</sub>, 232000), aldolase (M<sub>r</sub>, 158000), BSA (M<sub>r</sub>, 67000) and ovalbumin (M<sub>r</sub>, 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 pHCT79-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 BamHI 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
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 ± standard deviation obtained from at least three independent cultivations by two determinations per experiment. NG, No growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Malate synthase activity [U (mg protein)^{-1}]</th>
<th>MM + glucose</th>
<th>MM + acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DV21A05</td>
<td>&lt; 0.01</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>E. coli DV21A05 pHC79</td>
<td>&lt; 0.01</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>E. coli DV21A05(pAC1)</td>
<td>0.35±0.06</td>
<td>0.30±0.02</td>
<td>0.24±0.04</td>
</tr>
<tr>
<td>E. coli DV21A05(pAB-17)</td>
<td>0.29±0.04</td>
<td>0.24±0.04</td>
<td>0.24±0.04</td>
</tr>
<tr>
<td>E. coli DV21A05(pEKB1)</td>
<td>0.24±0.02</td>
<td>0.29±0.04</td>
<td>0.29±0.04</td>
</tr>
<tr>
<td>C. glutamicum WT</td>
<td>0.04±0.01</td>
<td>2.11±0.22</td>
<td>2.11±0.22</td>
</tr>
<tr>
<td>C. glutamicum(pEKB0)</td>
<td>0.04±0.01</td>
<td>2.32±0.13</td>
<td>2.32±0.13</td>
</tr>
<tr>
<td>C. glutamicum(pEKB1)</td>
<td>0.35±0.04</td>
<td>6.22±0.40</td>
<td>6.22±0.40</td>
</tr>
<tr>
<td>C. glutamicum ALB1</td>
<td>&lt; 0.01*</td>
<td>NG</td>
<td>NG</td>
</tr>
</tbody>
</table>

*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, pEKB1. 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 Smal-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 ninetofold higher specific malate synthase activity relative to C. glutamicum WT or its derivative carrying the cloning vector pEKO. When acetate minimal medium was used for growth, the specific malate synthase activities of C. glutamicum WT and C. glutamicum(pEKB0) 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
Malate synthase gene of *Corynebacterium glutamicum*

As shown in Fig. 1b, the 379 amino acid ORF codes for a polypeptide of 379 amino acids (Mr of 83,362) which was predicted to be the MalE protein. The predicted amino acid sequence of the malate synthase is shown below as RBS and a potential terminator structure is marked by arrows.

![Fig. 2. Nucleotide sequence of the 3024 bp Bfr-PvuII fragment carrying the aceB gene from *C. glutamicum*. The predicted amino acid sequence of the malate synthase is shown below as RBS and a potential terminator structure is marked by arrows.](image)

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 759 amino acids, malate synthase of other organisms consist of between 533 and 568 amino acids (for overview, 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 243 kb HpaI-NaeI fragment (Fig. 1b) carrying the *C. glutamicum* aceA gene encoding isocitrate lyase (Reinsheid et al., 1994). Comparison of the nucleotide sequence of this HpaI-NaeI fragment with that of the *aceB*-carrying Bfr-PvuII fragment revealed that both fragments share a common region, i.e. the first 504 nucleotides of the Bfr-PvuII 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.
To ensure that both genes are clustered on the C. glutamicum chromosome, Southern hybridization was performed. An aceB-specific 0.84 kb Smal- KpnI fragment and an aceA-specific 0.92 kb Asp700-BfiI fragment (see Fig. 1a and b) were used as probes. Hybridization of Asp700-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

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity [U (mg protein)⁻¹]</th>
<th>Recovery of activity (%)</th>
<th>Purification factor (×-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract*</td>
<td>2.3</td>
<td>667</td>
<td>415/4</td>
<td>6/2</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ultracentrifugation</td>
<td>18.0</td>
<td>308</td>
<td>408/2</td>
<td>13/3</td>
<td>98</td>
<td>2.1</td>
</tr>
<tr>
<td>MonoQ, pH 6.0</td>
<td>3.0</td>
<td>3.9</td>
<td>10/3</td>
<td>2/7</td>
<td>98</td>
<td>2.1</td>
</tr>
<tr>
<td>MonoQ, pH 8.0</td>
<td>2.0</td>
<td>3.5</td>
<td>10/1</td>
<td>2/8</td>
<td>98</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*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 40-fold to apparent homogeneity (Fig. 4), indicating that malate synthase represents about 20% of the cytoplasmic protein 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*ₚ of about 90,000.
(Fig. 4), which corresponds reasonably well to the M, predicted from the aceB sequence (82362, see above). By gel filtration the native M, of malate synthase was determined to be about 80,000. 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 Mg2+, Co2+ or Mn2+ could partially replace Mg2+, resulting in 25% and 15%, respectively, of the maximal activity. The Km values determined for acetyl CoA and glyoxylic acid were 12 μM and 30 μM, 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 glyoxylic acid, 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 (Ki) of 4.3 mM. No effect on enzyme activity was observed with fructose-1,6-bisphosphate, pyruvate, phosphoenolpyruvate, acetyl phosphate or 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 (Beice & Kornberg, 1968; Maloy & Nunn, 1982; Wilson & Maloy, 1987; Chung et al., 1988; Corr et al., 1992). The expression of this ace-operon is negatively controlled by the aceR 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, glycolic acid or allantoin (Vanderwinkel & De Vlieghere, 1968; Hattig 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, histidine H374 and aspartate D375 were identified as involved in the reaction mechanism (Alper 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 (H344 and D424 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.

**ACKNOWLEDGEMENTS**

We thank M. Romes for preparing the photographs and J. Carter-Siggloy for critical reading of the manuscript. This work was supported by grant BIOT-CT91-0264 (RZJE) from the EC-BRIDGE programme.

**REFERENCES**


---

3107


Received 13 May 1994; accepted 24 June 1994.