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NOTE

Ca^{2+} is Required to Make Functional Malate Synthase in *Corynebacterium glutamicum*

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The role of Ca^{2+} in making functional malate synthase in *Corynebacterium glutamicum* was investigated using the cloned DNA coding for the enzyme. Introduction of cloned *aceB* into *C. glutamicum* overexpressed malate synthase as judged by SDS-PAGE. However, the increase in enzyme activity of the expressed malate synthase did not match the level of overexpression observed in SDS-PAGE. Addition of Ca^{2+} to the growth medium specifically increased the activity. The malate synthase could be stained with ruthenium red in a Ca^{2+}-specific manner. This agrees with the previous observation which reported a potential Ca^{2+}-binding domain in the N-terminal region of the protein.

The glyoxylate bypass of *Corynebacterium glutamicum* and related species consists of two enzymes (12). Isocitrate lyase (ICL) encoded by *aceA* catalyzes the conversion of isocitrate, the TCA cycle intermediate, to glyoxylate and succinate (13). Malate synthase (MS), the product of *aceB*, catalyzes subsequent condensation of the glyoxylate with acetyl-CoA to produce malate, which in turn enters the TCA cycle (14). Expression of the glyoxylate bypass enzymes is essential for growth on two-carbon compounds, such as acetate, as the sole carbon sources, since it prevents the net loss of the acetate carbon as CO_2 in the TCA cycle (6).

The malate synthase of *C. glutamicum* is distinct from its counterparts in other organisms including that of *E. coli* (8, 14). The molecular weight of the malate synthase from *C. glutamicum*, which is 82,311 Daltons, is exceptional when compared with those of other malate synthases, which are approximately 60,000 Daltons (8). The unusual size is primarily due to approximately 150 amino acids located at the N-terminal region of the protein (8) (Fig. 1). This region, which shows no apparent amino acid sequence similarities with known malate synthases, also contains a stretch of conserved amino acids anticipated in Ca^{2+}-binding domains of EF-hand structure (7) widely found among eukaryotes (Fig. 1).

Previously, we isolated a *C. glutamicum aceB* clone by the complementation of *E. coli aceB* mutant (8). Among the clones, plasmid pSL08 overexpressed malate synthase in *C. glutamicum* (Fig. 2). We measured activities of malate synthase in the crude extracts which were made from cells grown on MB medium (4) containing 2% acetate (acetate is required to induce the malate synthase). The observed activities of malate synthase in the crude extracts made from cells harboring either pMT1 or pSL08 were 0.03 and 0.2 units (μmol min^{-1} mg^{-1}), respectively: plasmid pMT1 (2) carried no insert and was used as a control for the experiment. Although the difference in the amount of expressed malate synthase between these two extracts was approximately 40-fold as measured by densitometric scanning of the SDS-PAGE image, increase in the activity of the enzyme was only 7-fold. This suggests that either only a fraction of the expressed molecules is active or that each expressed molecule is partly active. Assuming that the inactive molecules of malate synthase may lack bound Ca^{2+}, we added calcium chloride to the growth medium and analyzed its effect on the activity of the protein. As shown in Fig. 3, addition of calcium chloride to the growth medium specifically increased the activity of the malate synthase. The maximal activity of 1.4 units was observed when 1.0 mM of calcium chloride was added to the growth medium. This corresponds to a 46 fold increase as compared to the basal level expression from the chromosomal gene. Addition of calcium chloride to the growth medium did not change the amount of expressed malate synthase (data not shown). Stimulation of the activity of malate synthase by calcium ion was not observed when plasmid pMT1 was introduced into the strain instead of plasmid pSL08 (Fig. 3). In addition, the activities of isocitrate lyase were not affected by the added calcium chloride to the growth medium (data not shown). Other cations, such as magnesium, manganese,

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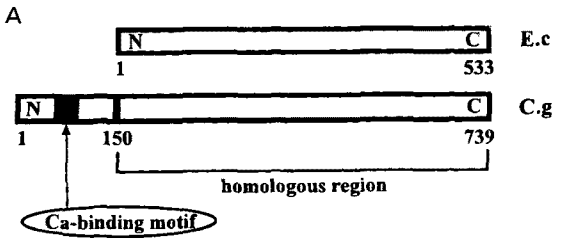

B

	n nn n X Y ZG I-X-Z n nn n
Caltractin (C)	EIKKMISEI DKDGSGTIDFEE FLTMMTAKM
Caltractin (Sd)	EIKKMIADI DKDGSGTIDFEE FLQMMTAKM
TroponinC	ELDAIIEEV DEDGSGTIDFEE FLVMMVRQM
Calbindin-32	DIEKVFSLY DRDNSGTIENEE LKGFLKDLL
Malate synthase (C.g)	ELQMLIDDY HRNNSGTIDQEA YEDFLKEIG

Fig. 1. Schematic representation of the *C. glutamicum* malate synthase and comparison of the putative calcium-binding motif of *C. glutamicum* malate synthase with Ca^{2+}-binding domains of *Chlamydomonas* (C) caltractin, frog skeletal muscle troponin C, *Drosophila melanogaster* calbindin, and *Scherffelia dubia* (Sd) caltractin.

(A) Schematic diagram showing the putative Ca^{2+}-binding domain of the malate synthase from *C. glutamicum* (C.g). The malate synthase of *E. coli* (E.c) was aligned to that of *C. glutamicum* on the basis of the amino acid sequence similarity (8). (B) Comparison of the putative Ca^{2+}-binding motifs. A typical structure of the EF-hand consists of approximately 30 amino acids where two perpendicularly oriented α-helixes flank a 12-residue calcium-binding loop (7). Each α-helix consists of uniformly-spaced core hydrophobic residues (labeled n). The central calcium-binding loop consists of oxygen-containing side chains (labeled X, Y, Z, -X, and -Z) which act as the ligands for bound Ca^{2+}. The glycine (labeled G), which is believed to form a sharp bend in the loop, and isoleucine (labeled I) are highly conserved among various Ca^{2+}-binding domains.

and copper, were ineffective in stimulating the malate synthase activity (data not shown). This clearly indicates that Ca^{2+} could be the limiting factor for the activity of the overexpressed malate synthases.

After observing that the malate synthase from *C. glutamicum* requires Ca^{2+} to be fully active, we investigated a possible binding of Ca^{2+} to the protein. This was done by using a Ca^{2+} specific dye, ruthenium red, which has been successfully used to identify Ca^{2+}-binding proteins on SDS-PAGE (1, 3). As shown in Fig. 4, malate synthase was specifically stained with ruthenium red dye on SDS-PAGE gels (Fig. 4, Panel B). The specificity of staining was demonstrated by competing the dye with Ca^{2+}. In the presence of 50 mM calcium chloride in the staining solution, almost no stained bands were observed (Fig. 4, Panel C). Addition of 50 mM magnesium ion, which is known to stimulate *C. glutamicum* malate synthase activity, did not prevent the protein from being stained with the dye (data not shown).

The data presented here indicate that the malate synthase of *C. glutamicum* is a Ca^{2+}-binding protein. There

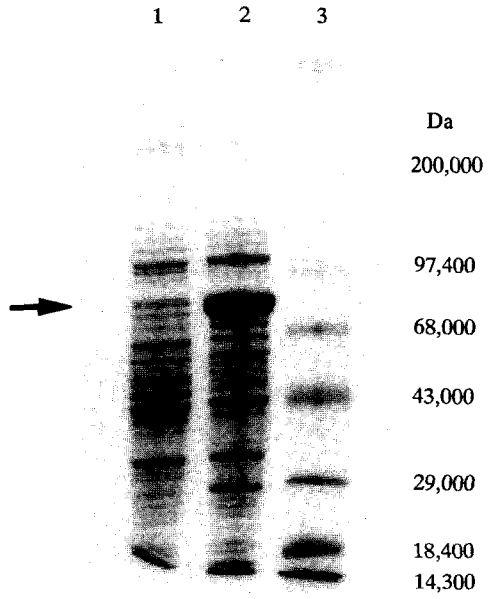

Fig. 2. Expression of malate synthase from plasmid pSL08.

Plasmid pSL08 carries the *aceB*-coding region and overexpresses the malate synthase (8). Plasmid pMT1 (2) carries no insert and used as a control for the experiment. Cells were grown in MB (4) containing 2% sodium acetate. Crude extracts were prepared as previously described (5). Arrow indicates the expressed malate synthase. Sizes of molecular weight markers are indicated. Lanes: 1, crude extract of *C. glutamicum* ASO19-E12 (8) harboring plasmid pMT1; 2, crude extract of *C. glutamicum* ASO19-E12 harboring plasmid pSL08; 3, molecular weight markers.

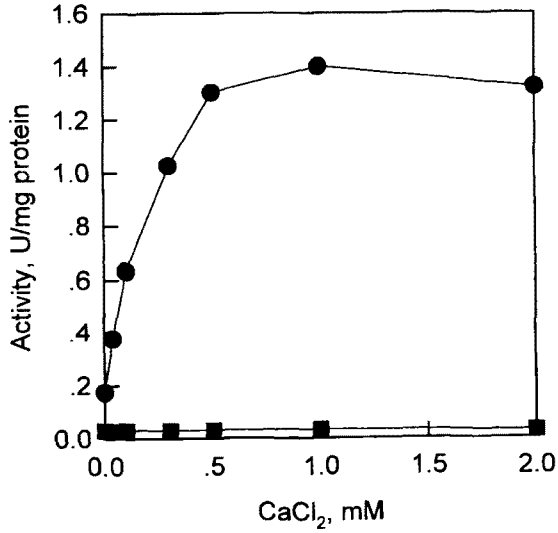

Fig. 3. Effect of added Ca^{2+} on the activity of malate synthase.

Various amount of calcium chloride was added to the growth medium as indicated. Cells carrying either pSL08 (●) or pMT1 (■) were grown in MB containing 2% sodium acetate (4). Crude extracts were prepared as previously described (5). Malate synthase was assayed as described (4).

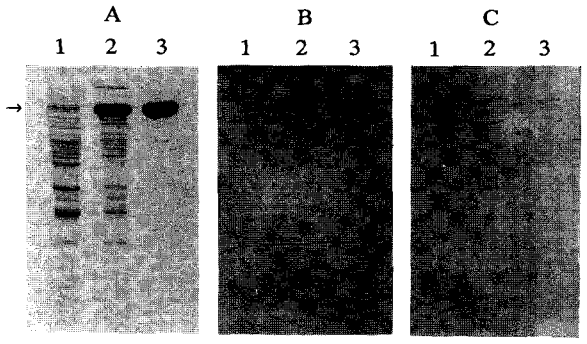

Fig. 4. Ruthenium red staining of malate synthase.
Crude extracts were prepared from cells grown on the MCGC medium containing 2% glucose or acetate (11). Ruthenium red staining was done as described (1). (A) SDS-PAGE analysis of the crude extract of *C. glutamicum* harboring plasmid pSL08 (lanes 1 and 2) and partially purified malate synthase (lane 3). Lanes: 1, MCGC glucose medium; 2, MCGC acetate medium; 3, partially purified malate synthase as described (14). (B) Ruthenium red staining of Panel A. (C) Ruthenium red staining of Panel A in the presence of 50 mM calcium chloride.

have been several reports on the requirement of Ca^{2+} in prokaryotes. Ca^{2+} is apparently involved in various cellular processes such as heat shock, pathogenicity, chemotaxis, differentiation, and the cell cycle (for reviews, see 9 and 10). Several calmodulin-like proteins were also identified in prokaryotes (10). The malate synthase of *C. glutamicum* is unique in having a domain which may bind Ca^{2+}. As shown in Fig. 1, the domain is mainly responsible for the protein's unusually large size. The malate synthase of *C. glutamicum* is an acidic protein with a pI value of 4.86 (8). The predicted pI of the N-terminal 160 amino acids is 4.37 which is also close to the known pI values of the many Ca^{2+}-binding proteins. The size of the N-terminal 160 amino acids (MW of 17,657) is also very similar to the known molecular weights of many Ca^{2+}-binding proteins. Although homologies exist between the known Ca^{2+}-binding proteins and the N-terminal domain of the malate synthase from *C. glutamicum*, direct demonstration of calcium binding would require investigations with purified protein.

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