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Biochemical characterisation of aconitase from *Corynebacterium glutamicum*

Meike Baumgart, Michael Bott*

Institut für Biotechnologie 1, Wilhelm-Johnen-Strasse, Forschungszentrum Jülich, D-52425 Jülich, Germany

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ABSTRACT

In this work, aconitase of the biotechnologically relevant microorganism *Corynebacterium glutamicum* was characterised. The specific activity of aconitase in extracts of glucose-grown cells was determined by four different assays. In three of them the formation or disappearance of *cis*-aconitate was measured, whereas in the fourth assay the aconitase reaction was coupled with isocitrate dehydrogenase. The highest activity was determined with *cis*-aconitate as substrate ($0.433 \pm 0.054\,U\,mg^{-1}$) and the lowest one with the coupled assay and citrate as substrate ($0.134 \pm 0.026\,U\,mg^{-1}$). Only the latter assay covers the complete aconitase reaction and thus gives the most relevant information on *in vivo* activity. For the determination of kinetic constants, aconitase was heterologously overproduced, purified, reactivated and biochemically characterised. Size exclusion chromatography indicated that the protein is monomeric. The enzyme showed Michaelis–Menten kinetics with K_m values of $480 \pm 200\,\mu M$ for citrate, $552 \pm 302\,\mu M$ for isocitrate and $18.5 \pm 3.4\,\mu M$ for *cis*-aconitate. The highest V_{max} was observed for the hydration of *cis*-aconitate with $40.6\,U\,mg^{-1}$. Aconitase was active over a wide pH and temperature range with maximal activity between pH 7.5 and 7.75 and at about 50 °C.

1. Introduction

Corynebacterium glutamicum is a non-pathogenic, predominantly aerobic, Gram-positive soil bacterium which was isolated because of its ability to excrete glutamate under certain conditions (Kinoshita et al., 1957). Nowadays it is the most important microorganism for large-scale biotechnological production of amino acids, predominantly L-glutamate (about 1.5 million tons per year) and L-lysine (about 850,000 tons per year) (Hermann, 2003). As the world market for amino acids is still increasing, there are ongoing efforts to improve production strains and production processes (Leuchtenberger et al., 2005). These efforts include systems biology approaches for the description of metabolism (Wendisch et al., 2006). Due to its industrial relevance, *C. glutamicum* has become a model organism of the *Corynebacterineae*, a suborder of the actinomycetes (Burkovski, 2008; Eggeling and Bott, 2005).

The tricarboxylic acid cycle (TCA cycle) is of central importance for the metabolism of *C. glutamicum* because it provides energy in the form of ATP and reducing equivalents for the respiratory chain as well as 2-oxoglutarate and oxaloacetate as biosynthetic precursors for the glutamate and aspartate family of amino acids (Bott, 2007; Bott and Niebisch, 2003). At the same time, the TCA cycle causes a loss of substrate carbon as carbon dioxide. Therefore, the flux through this cycle is an important aspect for the production of amino acids and consequently subject to intensive investigation (Bott, 2007).

The majority of enzymes of the TCA cycle of *C. glutamicum* have been characterised genetically and biochemically to some extent (Eikmanns, 2005), for instance citrate synthase (Eikmanns et al., 1994; Radmacher and Eggeling, 2007), isocitrate dehydrogenase (Bai et al., 1999; Chen and Yang, 2000; Eikmanns et al., 1995), the 2-oxoglutarate dehydrogenase complex (Niebisch et al., 2006; Usuda et al., 1996), succinate dehydrogenase (Kurokawa and Sakamoto, 2005), fumarase (Genda et al., 2006), malate dehydrogenase and malate:menaquinone oxidoreductase (Molenaar et al., 1998, 2000). The investigation of the regulation of the TCA cycle started only a few years ago but already led to the identification of a complex regulatory network at the level of gene expression (Bussmann et al., 2009; Emer et al., 2009; Krug et al., 2005; Wennerhold and Bott, 2006; Wennerhold et al., 2005) and at the posttranscriptional level by protein phosphorylation (Niebisch et al., 2006; Schultz et al., 2009). In particular the latter regulatory mechanism proved to be highly important for glutamate production (Schultz et al., 2007).

Aconitase is the second enzyme of the TCA cycle and catalyses the stereospecific and reversible isomerisation of citrate to isocitrate with *cis*-aconiate as intermediate (for review see Beinert et al., 1996). It contains a [4Fe–4S] cluster where one of the four iron ions is not bound to the protein backbone via a cysteine residue. This iron is crucial for enzymatic activity and is easily lost under oxidative stress conditions resulting in an inactive enzyme. Aconitase is not only part of the TCA cycle, but also of the glyoxylate cycle, which

* Corresponding author. Tel.: +49 2461 613294; fax: +49 2461 612710.
E-mail address: m.bott@fz-juelich.de (M. Bott).

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serves as anaplerotic reaction during growth on acetate, ethanol or fatty acids, and of the methylcitrate cycle, which is responsible for the catabolism of propionate and odd-numbered fatty acids (Bott, 2007). In eukaryotes and some prokaryotes, aconitase also has a regulatory function by binding to certain mRNAs and inhibiting or increasing their translation (Alen and Sonenshein, 1999; Banerjee et al., 2007; Beinert and Kiley, 1999; Tang and Guest, 1999; Tang et al., 2002, 2004).

The presence of an aconitase in *C. glutamicum* cell extract was already shown 50 years ago (Shiio et al., 1959). Sequencing of the genome of the *C. glutamicum* type strain ATCC13032 (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003) led to the identification of a single gene coding for aconitase (*acn*, cg1737, NCgl1482). It encodes a protein of 943 amino acid residues with a calculated molecular mass of 102,168 Da (Schaffer et al., 2001). It shows 59% sequence identity to AcnA of *Escherichia coli* and 47% sequence identity to human IRP1 (iron regulatory protein 1) and thus belongs to family II within the aconitase superfamily (Gruer et al., 1997a; Makarova and Koonin, 2003; Schinko et al., 2009). The three cysteine residues which coordinate the [4Fe–4S] cluster are located at positions 479, 545 and 548. Expression of the aconitase gene is influenced by at least three different transcriptional regulators, namely AcnR (Krug et al., 2005), RipA (Wennerhold et al., 2005) and RamA (Emer et al., 2009). Whereas the physiological role of *acn* repression by AcnR is not yet clear, repression by RipA is part of the iron homeostasis and serves to reduce the iron demand under iron limitation (Frunzke and Bott, 2008). Activation of *acn* transcription by RamA serves to allow increased flux through the TCA cycle during growth on acetate (Cramer et al., 2006; Wendisch et al., 2000).

In this study, we have compared different aconitase assays and performed the first biochemical characterisation of aconitase from *C. glutamicum*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

Table 1 lists all bacterial strains and plasmids used in this study. *E. coli* was grown at 37 °C in LB broth (Sambrook et al., 1989). Kanamycin (50 μg ml^{-1}) or ampicillin (100 μg ml^{-1}) were added as appropriate. *C. glutamicum* was grown aerobically at 30 °C and 120 rpm in 500-ml baffled shake flasks containing 50 ml medium. The cells were cultivated either in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI, USA) supplemented with 4% (w/v) glucose or in CGXII minimal medium (Keilhauer et al., 1993) containing 4% (w/v) glucose as carbon and energy source and 30 mg l^{-1} 3,4-dihydroxybenzoate as iron chelator.

2.2. Cultivation of cells and preparation of protein extracts for the determination of the specific aconitase activity

A preculture of 20 ml BHI medium supplemented with 4% (w/v) glucose was inoculated from a fresh agar plate and grown at 30 °C for 8 h. The cells were harvested, washed once with PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4 with HCl) and resuspended in 5 ml PBS. 50 ml CGXII medium with 4% (w/v) glucose was inoculated with these cells to an initial OD_{600} of 1 and incubated for 16 h at 30 °C. These cells were used to inoculate the main culture (50 ml CGXII with 4% (w/v) glucose) to an initial OD_{600} of 1. When the culture had reached an OD_{600} of about 5, the cells were harvested, washed once with PBS and stored at −20 °C until they were used for enzyme assays.

For the preparation of protein extracts the cells were thawed, resuspended in 1 ml 100 mM Tris–HCl pH 8.0 containing Complete EDTA-free protease inhibitor (Roche Diagnostics) as recommended by the manufacturer. The cell suspension was mixed with 250 mg zirconia/silica beads (0.1 mm diameter, Biospec, Bartlesville, USA) in a 2 ml Eppendorf tube and mechanically disrupted by 3× 30 s shaking in a Silamat S5 (Ivoclar Vivadent, Ellwangen, Germany). Cell debris and unbroken cells were separated by 2× 15 min centrifugation at 16,000 × *g* at 4 °C. Between the two centrifugation steps the supernatant was transferred to a fresh tube. The resulting cell extract was kept on ice and immediately used for enzyme assays.

2.3. Aconitase enzyme assays

The activity of aconitase was measured by monitoring the formation or disappearance of *cis*-aconitate at 240 nm in a UV-1601PC spectrophotometer (Shimadzu, Duisburg, Germany) (for the determination of the temperature dependency) or in a V-560 UV/VIS spectrophotometer (Jasco, Gross-Umstadt, Germany) (for all other assays) using an extinction coefficient for *cis*-aconitate of 3.5 mM^{-1} cm^{-1}. One unit (U) of aconitase activity is defined as 1 μmol *cis*-aconitate formed or converted per minute. Unless otherwise stated, the assays were performed at 30 °C in 100 mM Tris–HCl, pH 8.0.

The specific activity of aconitase in cell extracts was determined with all three substrates (trisodium citrate and D,L-isocitrate: 20 mM; *cis*-aconitate: 0.2 mM) in 100 mM Tris–HCl pH 8.0 using 10 μl cell extract (1:2 diluted for *cis*-aconitate). In addition, a coupled assay with isocitrate dehydrogenase was performed in which the formation of NADPH at 340 nm (ε = 6.27 mM^{-1} cm^{-1}) was monitored. The reaction mixture contained 100 mM Tris–HCl pH 8.0, 20 mM trisodium citrate, 1 mM $NADP^+$, 1 mM $MnSO_4$ and 1.6 U isocitrate dehydrogenase (USB Corporation, Cleveland, OH, USA). In this

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
DH5α	F$^-$ ϕ80*dlac*Δ(*lacZ*)M15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (r_K^-, m_K^+) *deoR thi-1 phoA supE44* λ$^-$ *gyrA96 relA1*; strain used for cloning procedures	Hanahan (1983)
BL21(DE3)	*ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* λ(DE3); strain used for aconitase overproduction	Studier and Moffatt (1986)
C. glutamicum		
ATCC13032	biotin-auxotrophic wild type	Kinoshita et al. (1957)
Plasmids		
pET16b	Amp^R; vector for overexpression of genes in *E. coli*, adding an N-terminal decahistidine affinity tag and a factor Xa cleavage site to the synthesised protein	Novagen
pET16b-acn	Amp^R; pET16b derivative coding for *C. glutamicum* aconitase with an N-terminal decahistidine tag	This work
pTYB2	Amp^R; vector for overproduction of proteins fused to an intein tag	New England Biolabs
pTYB2-acn	Amp^R; pTYB2 derivative coding for *C. glutamicum* aconitase C-terminally fused to an intein tag	This work