A spectrophotometric assay for measuring acetyl–coenzyme A carboxylase

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Article history:
Received 18 October 2010
Received in revised form 24 November 2010
Accepted 30 November 2010
Available online 5 December 2010

Abstract

Acetyl–coenzyme A (CoA) carboxylase catalyzes the first step in the biosynthesis of fatty acids in bacteria and eukaryota. This enzyme is the target of drug design for treatment of human metabolic diseases and of herbicides acting specifically on the eukaryotic form of the enzyme in grasses. Acetyl–CoA carboxylase activity screening in drug and herbicide design depends mostly on a time-consuming enzyme assay that is based on the incorporation of radiolabeled bicarbonate into the product malonyl–CoA. Here we describe a new simple, continuous, and quick photometric assay avoiding radioactive substrate. It couples the carboxylation of acetyl–CoA to the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of malonyl–CoA, which is catalyzed by recombinant malonyl–CoA reductase of Chloroflexus aurantiacus. This assay can be adapted for high-throughput screening.

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Materials and methods

Chemicals were obtained from Amersham Biosciences (Freiburg, Germany), Fluka (Neu-Ulm, Germany), Sigma–Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), Serva

Abbreviations used: ACC, acetyl–coenzyme A carboxylase; ATP, adenosine triphosphate; CoA, coenzyme A; MCR, malonyl–CoA reductase; NADPH, nicotinamide adenine dinucleotide phosphate; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; LB, Luria Broth; DTT, dithiothreitol; Na2EDTA, disodium ethylenediamine tetra acetate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UV, ultraviolet; Mops, 3-(N-tris(hydroxymethyl)methyl)glycine.
(Heidelberg, Germany), and Roth (Karlsruhe, Germany). Biochemicals were obtained from Roche Diagnostics (Mannheim, Germany), Applichem (Darmstadt, Germany), and Gerbu (Craiberg, Germany). Materials for cloning and expression were purchased from MBI Fermentas (St. Leon-Rot, Germany), New England Biolabs (Frankfurt, Germany), Novagen (Schwalbach, Germany), Finnzymes (Espoo, Finland), MWB Biotech (Ebersberg, Germany), Biomers (Ulm, Germany), and Qiagen (Hilden, Germany). Materials and equipment for protein purification were obtained from GE Healthcare (Freiburg, Germany) and Millipore (Eschborn, Germany).

Syntheses

Acetyl–CoA was synthesized from acetic acid anhydride [20].

Organisms and growth conditions

Genomic DNA used as polymerase chain reaction (PCR) template originated from C. aurantiacus OK-70-fl (DSM 636). Metallosphaera sedula TH2 (DSM 5348) was grown autotrophically at 75 °C on a chemically defined medium (pH 2.0) under gassing with a mixture of 19% CO2, 3% O2, and 78% H2. Escherichia coli strain DH5α was grown at 37 °C in Luria Broth (LB). Ampicillin was added to E. coli cultures to concentrations of 50–100 µg/ml. Zea mays (Pioneer PR38A24, Buxtehude, Germany) was grown in a greenhouse in normal potting soil using a 16:8-h day (25 °C) cycle. The plants were harvested after 10 days, and the upper parts of the leaves were collected for further use.

Preparation of cell extracts

Mixer mill

Cells of E. coli DH5α and M. sedula were suspended in a 3-fold volume of 50 mM tris(hydroxymethyl)amino methane/HCl (Tris–HCl) buffer (pH 7.8) containing 1 mM MgCl2. The cell suspension was treated in a mixer mill (type MM2, Retsch, Haare, Germany) for 10 min at 30 Hz. The supernatant obtained after centrifugation (10 min, 16,000 g, 4 °C) was kept for further use.

French press

Cells of E. coli DH5α and M. sedula were suspended in 1–3 volumes of 50 mM Tris–HCl (pH 7.8) containing 1 mM MgCl2. After the addition of Dnase I (1 mg/ml), the cell suspension was passed twice through a chilled French pressure cell at 137 MPa. The cell lysate was centrifuged for 1 h at 100,000 g (4 °C), and the supernatant was kept for further use.

Extract from corn leaves

The upper parts of the leaves were cut into small pieces. The leaf pieces were homogenized at 4 °C in a blender using 100 mM N-tris(hydroxymethyl)methylglycine/KOH (Tricine–KOH) buffer (pH 8.3) containing 300 mM glycerol, 5 mM dithiothreitol (DTT), 2 mM disodium ethylene diamine tetra acetate (Na2EDTA), 0.5 mM phenyl methane sulfonil fluoride, and 0.01% (v/v) Triton X-100. The homogenate was filtrated through a double layer of gauze and then centrifuged for 20 min at 30,000g (4 °C). The supernatant was kept for further use.

Cloning and heterologous expression

Standard protocols were used for purification, preparation, cloning, transformation, and amplification of DNA [21–23]. Plasmid DNA was isolated with the QiNaprep Spin Miniprep Kit (Qiagen). For amplification of the MCR gene from C. aurantiacus chromosomal DNA, two oligonucleotides were used binding upstream (5’-ctagccctttayaagggaaaggaaggggayaagggag 3’) and downstream (5’-aggattctacaaggggaaggggaagggggaagggag 3’). Primers also included a sequence encoding a Strep-tag II. PCR was performed with Phusion DNA polymerase (Finnzymes) for 32 cycles, including denaturation for 20 s at 98 °C, annealing for 30 s at 60 °C, polymerization for 150 s at 72 °C, and a final extension at 72 °C for 10 min. The PCR product was purified, restricted, and cloned into a pTrc99A vector. Competent E. coli DH5α cells were transformed with the plasmid and grown at 37 °C in 1- to 10-L cultures of LB medium with 100 µg ampicillin ml⁻¹. At OD600 = 0.6 to 0.8, the expression was induced with 0.5 mM isopropyl thiogalactopyranoside. The cultures were harvested after additional growth for 4 h at 37 °C and stored at −80 °C until use.

Purification of recombinant MCR from E. coli cell extract

Extracts from E. coli cells containing the recombinant MCR were heat precipitated for 15 min at 60 °C, followed by centrifugation (16,000g) at 4 °C. The supernatant was applied at a flow rate of 2 ml min⁻¹ to a 20-ml Strep-Tactin column (IBA, Göttingen, Germany) that had been equilibrated with 5 column volumes of 100 mM Tris–HCl (pH 8.0) containing 150 mM NaCl (buffer A). The column was washed with 5 column volumes of buffer A. The recombinant enzyme was eluted with buffer A containing 2.5 mM dethiobiotin. Fractions of 2 ml were collected and tested for MCR activity. Active elution fractions were pooled and concentrated by ultrafiltration with an Amicon (10 kDa) membrane (Millipore). The enzyme was stored in the presence of 20% glycerol at −20 °C.

Enrichment of ACC from corn leaves extract

ACC from extract of leaves (30,000 g supernatant) was enriched by ammonium sulfate precipitation. All steps were performed at 4 °C. At first, the concentration of ammonium sulfate was slowly increased to 30% saturation, followed by stirring for 20 min. After centrifugation (20,000g), the supernatant was subjected to another precipitation step (40% saturation), and the suspension was centrifuged again. The supernatant was discarded, and the pellet was redissolved in extraction buffer (see “Preparation of cell extracts”). The suspension (2.5 ml) was then applied to an 8.5-ml PD10 desalting column (GE Healthcare), which was developed with

25 ml of 100 mM Tricine–KOH (pH 8.0) containing 500 mM glycerol, 0.5 mM DTT, 2 mM Na₂EDTA, and 50 mM KCl. The column was washed with 3.5 ml of the same buffer to elute desalted ACC.

**Enrichment of ACC from M. sedula cell extract**

Extract (100,000g supernatant) of 5 g of *M. sedula* cells (5 ml) was applied at a flow rate of 1 ml min⁻¹ to a 6-ml DEAE–Sephacel column (GE Healthcare), which was equilibrated with 30 ml of 20 mM triethanolamine–NaOH (pH 7.8) (buffer B). After 40 min, a washing step with 50 mM NaCl in buffer B was performed. The NaCl concentration was subsequently increased by 25-mM steps for elution. ACC activity eluted at 100 mM NaCl, and all active fractions were pooled and concentrated by ultrafiltration with an Amicon (10 kDa) membrane (Millipore).

**Enzyme assays**

All photometric measurements were performed using a 500-μl cuvette with a 1-cm pathlength.

**MCR**

The assay mixture (300 μl) contained 100 mM Mops–KOH (pH 7.8), 2 mM MgCl₂, 4 mM ATP, 0.4 mM NADPH, and recombinant MCR. The reaction was started by the addition of 0.3 mM malonyl–CoA and was carried out at 57°C. NADPH oxidation was monitored at 365 nm (ε₃₆₅nm = 3.4 × 10⁴ M⁻¹ cm⁻¹). For determination of the activity depending on temperature of this reaction, the rates of catalysis were measured from 24 up to 77°C.

**Spectrophotometric ACC assay**

For *M. sedula*, the assay mixture (300 μl) contained 100 mM Mops–KOH (pH 7.8), 5 mM MgCl₂, 4 mM DTT, 10 mM NaHCO₃, 5 mM MgCl₂, 4 mM ATP, 0.4 mM NADPH, and enriched ACC. The reaction (at 60°C) was started by the addition of 0.4 mM acetyl–CoA. In the case of *Z. mays*, the mixture (300 μl) contained 100 mM Mops–KOH (pH 8.0), 5 mM MgCl₂, 4 mM DTT, 15 mM NaHCO₃, 2 mM ATP, 0.4 mM NADPH, 5 μg MCR, and enriched ACC. The reaction (at 40°C) was started by the addition of 2 mM acetyl–CoA. In both cases, the oxidation of NADPH was monitored at 365 nm.

**[14C]NaHCO₃ incorporation assay**

The assay mixture (300 μl) had the same composition as for the respective photometric assay. Unlabeled bicarbonate was substituted by [14C]bicarbonate with a specific radioactivity of 7.4 Bq nmol⁻¹. Samples of 100 μl were withdrawn after 1, 2, and 5 min of incubation, and the reaction was stopped with 20 μl of 6 M HCl. All samples were agitated room temperature for 12 h to remove all nonincorporated radioactive CO₂. Residual radioactivity was quantified by liquid scintillation counting.

**Testing for sulfydryls**

To check the requirement of reduced cysteines for the activity of the recombinant MCR, the enzyme was preincubated (~20 min) in assay mixture (see above) containing either 10, 50, 100, or 250 μM N-ethylmaleimide. The reaction was started by the addition of malonyl–CoA. The influence of DTT was tested at a final concentration of 3 mM in the assay mixture.

**Other methods**

DNA and amino acid sequences were analyzed with the BLAST network service at the NCBI (National Center for Biotechnology Information, Bethesda, MD, USA) and using the program Clone Manager 7 (SciEd Software, Cary, NC, USA). Protein concentrations were determined by the Bradford method [24] using bovine serum albumin as a standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 12.5%) was performed by the Laemmli method [25]. Proteins were visualized by Coomassie Brilliant Blue R-250 staining [26].

**Deposition of strain and vector sequence**

*C. aurantiacus* strain OK-70–H is available at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) under the accession number DSM 636. The complete sequence of the expression vector is provided in the supplementary material. The plasmid can be provided by the authors at request.

**Results and discussion**

**Cloning and expression of MCR**

The bifunctional MCR catalyzes the following reaction: malonyl–CoA⁻ + 2NADPH + 2H⁺ → 3-hydroxypropionate⁻ + 2NAD⁺ + CoA. The free energy change associated with this reaction is approximately −12 kJ reaction⁻¹, and the equilibrium constant Kₑq (pH 7) is approximately 130 M⁻¹. The MCR *Caur_2614* gene (3.7 kb) of *C. aurantiacus* codes for a 132-kDa protein (1220 amino acids), which has a Vₘₐₓ of 10 μmol min⁻¹ ng⁻¹ (55°C), a catalytic number of 50 s⁻¹, apparent Kₘ values of 30 μM malonyl–CoA and 25 μM NADPH, and a pH optimum of 7.8 with half-maximal activities at pHs 6.5 and 8.5. Activity depends on the presence of divalent cations such as Mg²⁺ (2 mM) [18]. This gene was amplified by PCR and cloned into the expression vector pTrc99A, resulting in a plasmid (pTrc–McrCa) encoding an N-terminal Strep-tagged enzyme with an altered molecular mass of approximately 133 kDa. The plasmids were transformed into *E. coli* DH5α for expression. The gene was heterologously expressed and the corresponding enzyme was soluble, as deduced from an induced protein band at around 130 kDa in SDS–PAGE of cell extract (Fig. 2).

**Purification and properties of MCR**

*E. coli* cell extract (100,000g supernatant) containing the recombinant enzyme was heated for 15 min at 60°C; the enzyme from the moderate thermophilic *C. aurantiacus* remained active and soluble. The supernatant was then applied to a Strep-Tactin affinity column. The supernatant was then applied to a Strep-Tactin affinity column. The column was washed with 3.5 ml of the same buffer to elute desalted ACC.

**Fig. 2.** Denaturing PAGE (8%) of fractions obtained during the purification of heterologously expressed MCR of *C. aurantiacus* from 3 g of *E. coli* cells. Lane 1: extract (100,000g supernatant) of induced *E. coli* cells (20 μg); lane 2: heat precipitation fraction (10 μg); lane 3: affinity chromatography fraction (7 μg protein). The gel was stained with Coomassie Brilliant Blue R-250.
The catalytic activity of MCR was determined after storage with different concentrations of glycerol, different storage periods, and different temperature ranges. The best results were achieved with a final concentration of 20% (v/v) glycerol. Under these conditions, the enzyme was stable for several months at –20 °C and for at least 2 weeks at 4 °C. Glycerol also prevented precipitation of concentrated enzyme (above 2 mg/ml). However, repeatedly thawing and freezing gradually inactivated the enzyme.

MCR was also tested for catalytically active cysteine residues by preincubation with N-ethylemaleimide, which specifically attacks sulfhydryl moieties [27]. No significant change of enzyme activity was observed. DTT-stabilizing sulfhydryl groups had no influence either. In addition, alignments of orthologous enzymes from other species did not reveal any conserved cysteines. Therefore, the use of reducing agents during storage seems to be unnecessary.

The optimal growth temperature of C. aurantiacus is 55 °C. The recombinant MCR was most active at 57 °C but remained active over a broad temperature range (Fig. 3), with half-maximal activities at 38 and 69 °C. To ensure that only malonyl–CoA served as substrate for MCR, we tested methylmalonyl–CoA as well. Succinyl–CoA and propionyl–CoA were tested previously [18]. Indeed, MCR did not catalyze the reduction of the other compounds. The enzyme was specific for NADPH.

Enrichment and activity measurements of ACC from corn leaves and M. sedula

ACC catalyzes the following reaction: acetyl–CoA + HCO₃⁻ + ATP → malonyl-CoA + H₂O + ADP + P₇ (where ADP is adenosine diphosphate and P₇ is inorganic phosphate). The free energy change associated with this reaction is approximately –14 kJ reaction⁻¹, and the equilibrium constant Kₑq (pH 7) is approximately 280 M⁻¹. This reaction also requires Mg²⁺. We used the top parts of corn leaves for enrichment of plastidic ACC, the target for herbicides.

Raw extract was precipitated with ammonium sulfate, and the final fraction was desalted using a PD-10 column to get rid of flavonoids, which could interfere with the photometric test.

Activity of ACC was measured at 40 °C using both the coupled photometric assay (Fig. 4) and the standard assay for incorporation of [¹⁴C] bicarbonate. The assays were performed on the same day and with the same buffer and stock solutions for comparison. The photometric assay resulted in 67 nmol min⁻¹ (mg protein⁻¹), whereas the radioactive assay yielded 49 nmol min⁻¹ (mg protein⁻¹).

In addition, ACC from the Archaeon M. sedula (order of Sulfolobales) was enriched from extracts of autotrophically grown cells by the use of an anion exchange column (DEAE–Sephacel). Autotrophy in M. sedula proceeds via the 3-hydroxypropionate/4-hydroxybutyrate cycle [28]. This cycle also makes use of ACC [29,30] and MCR [31]. The archaeal MCR, however, catalyzes only the formation of malonic semialdehyde and differs from the Chloroflexus enzyme. Endogenous MCR activity of M. sedula was removed during the enrichment of ACC. Again, both kinds of assay (at 60 °C) were used to determine the ACC activity. In this case, the photometric assay (Fig. 4) yielded 57 nmol min⁻¹ (mg protein⁻¹) compared with 46 nmol min⁻¹ (mg protein⁻¹) observed with the radioactive assay.

To verify the proper functioning of the photometric assay under the applied test conditions, we performed a series of controls where one of the components (acetyl–CoA, ATP, MCR, or ACC fraction) was omitted. None of these controls resulted in an oxidation of NADPH that was higher than the normal background. The overall stoichiometry was observed to be 1.8 mol of NADPH oxidized per mol of acetyl–CoA added to the assay, which is close to the expected ratio of 2:1 if the reaction went to completion. The equilibrium of the reduction of malonyl–CoA to malonic semialdehyde with NADPH is in vivo far on the side of the aldehyde. Because the reduction of the intermediate malonic semialdehyde to 3-hydroxypropionate is not 100%, a stoichiometry of approximately 1.9 mol of NADPH oxidized per acetyl–CoA carboxylated to malonyl–CoA may be used. Because all assays were performed under aerobic conditions, the buffers and stock solutions were equilibrated with CO₂. Therefore, the omission of bicarbonate had no strong effect on the assay.

**Assay conditions**

Coupled spectrophotometric assays require the coupling enzyme activity to be present in approximately a 10 times higher amount compared with the enzyme activity to be measured (see Fig 4). Spectrophotometric assays used to determine the specific enzyme activity of ACC fractions. MCR was used as a coupling enzyme. Malonyl–CoA formed by the carboxylation of acetyl–CoA was reduced to 3-hydroxypropionate under consumption of 2 NADPH, which was followed spectrophotometrically at 365 nm. The reaction was linear for several minutes. (A) ACC of maize was measured at 40 °C, and 5 μg of MCR was used. (B) ACC of M. sedula was measured at 60 °C, and 3.5 μg of MCR was used. For detailed assay conditions, see Materials and Methods.
The effect of the relative amount of coupling enzyme MCR on the spectrophotometric assay of ACC from maize at 40 °C (MCR 5 µg MCR/0.3 ml assay) was kept constant, whereas the amount of ACC was varied. MCR activity (34 mU) refers to the assay temperature at 40 °C. Note that according to the rules of coupled enzyme assays, MCR must be present in at least 10-fold excess (mU ACC/mU MCR <0.1) to guarantee the linearity of the assay. For assay conditions, see Materials and Methods.

**Fig. 5.** Effect of the relative amount of coupling enzyme MCR on the spectrophotometric assay of ACC from maize at 40 °C. MCR (5 µg MCR/0.3 ml assay) was kept constant, whereas the amount of ACC was varied. MCR activity (34 mU) refers to the assay temperature at 40 °C. Note that according to the rules of coupled enzyme assays, MCR must be present in at least 10-fold excess (mU ACC/mU MCR <0.1) to guarantee the linearity of the assay. For assay conditions, see Materials and Methods.

**Acknowledgment**

This work was supported by Deutsche Forschungsgemeinschaft.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jab.2010.11.046.

**References**
