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A capillary electrophoretic assay for acetyl coenzyme A carboxylase

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ABSTRACT

A simple off-column capillary electrophoretic (CE) assay for measuring acetyl coenzyme A carboxylase holoenzyme (holo-ACC) activity and inhibition was developed. The two reactions catalyzed by the holo-ACC components, biotin carboxylase (BC) and carboxyltransferase (CT), were simultaneously monitored in this assay. Acetyl coenzyme A (CoA), malonyl-CoA, adenosine triphosphate (ATP), and adenosine diphosphate (ADP) were separated by capillary electrophoresis, and the depletion of ATP and acetyl-CoA as well as the production of ADP and malonyl-CoA were monitored. Inhibition of holo-ACC by the BC inhibitor, 2-amino-*N*,*N*-dibenzyloxazole-5-carboxamide, and the carboxyltransferase inhibitor, andrimid, was confirmed using this assay. A previously reported off-column CE assay for only the CT component of ACC was optimized, and an off-column CE assay for the BC component of ACC also was developed.

Acetyl coenzyme A carboxylase (ACC)[1] catalyzes the first committed and rate-limiting step in fatty acid biosynthesis in all bacteria, plants, and animals. The biotin-dependent carboxylation of acetyl coenzyme A (CoA) to form malonyl-CoA is carried out in two half-reactions (Fig. 1) [1]. In the first half-reaction, biotin carboxylase (BC) catalyzes the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and the carboxylation of biotin, which in vivo is covalently attached to biotin carboxyl carrier protein (BCCP). In the second half-reaction, carboxyltransferase (CT) catalyzes the transfer of the carboxyl group on biotin to acetyl-CoA to form malonyl-CoA. In bacteria, BC, CT, and BCCP are separate proteins, and BC and CT retain their activity using free biotin in place of BCCP [1]. In eukaryotes, these three components of ACC are combined in one polypeptide [1]. ACC is a target for the development of antibiotics [2,3], herbicides [4], and therapeutic agents for metabolic syndrome (including obesity and type 2 diabetes) [5–9] and cancer [10,11]. Thus, effective assays to measure the inhibition of ACC are desirable.

Several assays have been developed to monitor the activity of isolated bacterial BC and CT, as well as ACC holoenzyme (holo-ACC), from either bacterial or eukaryotic sources. Hereafter, holo-ACC refers to the case when all three bacterial proteins—BC, CT, and BCCP—are present in solution. Early studies of *Escherichia coli* ACC used a radiolabeled bicarbonate fixation assay for determining the activity of holo-ACC and isolated BC [12]. Moreover, BC was also assayed with an enzyme-coupled assay, where ADP formation was monitored spectrophotometrically at 340 nm via NADH oxidation [12]. The enzymatic activity of isolated bacterial CT was commonly assayed in the reverse (nonphysiological) direction either using a radioactivity assay that measured the biotin-dependent decarboxylation of radiolabeled malonyl-CoA or where acetyl-CoA production was coupled to the citrate synthase–malate dehydrogenase reaction and NAD^+ reduction was monitored spectrophotometrically at 340 nm [12]. More recently, Kroeger and coworkers developed an NADPH-based spectrophotometric assay for holo-ACC, where malonyl-CoA reductase was used to catalyze the NADPH-dependent reduction of the product, malonyl-CoA [13]. In addition, Alves and coworkers reported an assay for holo-ACC based on liquid chromatography with tandem mass spectrometry (LC–MS/MS), where the enzyme and substrates were incubated for 3 h at room temperature and malonyl-CoA formation was monitored [14].

Several of these assays for ACC have been adapted and optimized for high-throughput screening. Harwood and coworkers [7] developed a plate reader-based high-throughput screening assay for mammalian ACC based on the radiolabeled bicarbonate fixation assay of Guchhait and coworkers [12]. Subsequently, Santoro and coworkers developed a plate reader-based high-throughput screening assay for bacterial CT in the nonphysiological direction where citrate synthase was coupled to the production of acetyl-CoA and the product CoA was detected with DTNB (Ellman's reagent) [15]. Soriano and coworkers developed a plate reader-based high-throughput assay for ACC that involved the

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[1] *Abbreviations used:* ACC, acetyl coenzyme A carboxylase; CoA, coenzyme A; BC, biotin carboxylase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; BCCP, biotin carboxyl carrier protein; CT, carboxyltransferase; holo-ACC, ACC holoenzyme; LC, liquid chromatography; MS/MS, tandem mass spectrometry; UV, ultraviolet; CE, capillary electrophoresis; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.

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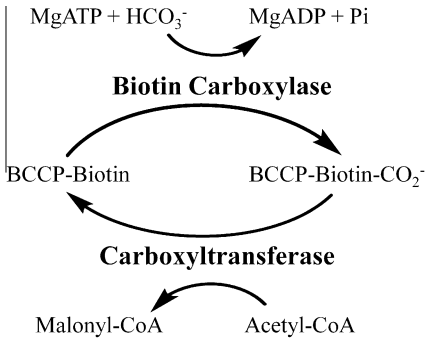

Fig.1. ACC reaction scheme. Pi, inorganic phosphate.

use of ^{32}P-labeled ATP [16]. Seethala and coworkers developed a high-throughput assay for mammalian ACC where radiolabeled acetyl-CoA was used in an ACC/fatty acid synthase-coupled assay and radiolabeled palmitic acid was detected [17]. Liu and coworkers reported two high-throughput assays for mammalian ACC where Amplex Red was used with two coupled enzyme-catalyzed reactions to detect phosphate by fluorescence or fluorescence polarization was used to monitor fluorescently labeled ADP binding to an anti-ADP antibody [18]. Chung and coworkers reported a high-throughput fluorescence assay where mammalian ACC activity was coupled to fatty acid synthase activity [19]. Recently Alves and coworkers developed a plate reader luminescence-based assay for assaying holo-ACC that monitored the depletion of ATP [14].

The previously developed assays all have significant limitations for screening of ACC inhibitors. The assays based on radiolabeling can be expensive and complex in practice due to health, waste, and regulatory issues. The method based on LC–MS/MS provides comparable selectivity without the health and regulatory issues associated with radiolabeling, but it relies on complex methodology and expensive instrumentation. Assays based on enzyme-coupled reactions are inherently indirect and more susceptible to false positives for inhibition studies. In addition, spectrophotometric assays based on ultraviolet (UV) absorbance or fluorescence with UV excitation are prone to spectral interference by inhibitors and biological sample matrices.

Recently, we developed a simple capillary electrophoresis-based assay for the CT component of ACC using UV absorbance detection [20]. This method was developed to overcome spectral interference for particular inhibitors and botanical extracts that could not be assayed using the enzyme-coupled spectrophotometric method employed for the rest of the study [21]. Capillary electrophoresis has been used for a wide array of applications and has several advantages, including high separation efficiency, simplicity, small sample volume (nanoliters), and rapid analysis time [22]. It has been demonstrated that capillary electrophoresis can be used effectively for performing quantitative enzyme assays and studying enzyme inhibition [23,24]. Because capillary electrophoresis is an electrophoretic separation technique, spectral interference is reduced by separation of substrates, products, inhibitors and sample matrix components.

Here we report an off-column capillary electrophoresis (CE) assay to measure holo-ACC activity and inhibition that allows for simultaneous monitoring of the BC- and CT-catalyzed reactions. To our knowledge, this is the first report of simultaneous direct monitoring of ATP and acetyl-CoA depletion and ADP and malonyl-CoA production for determination of holo-ACC activity. In addition, the CE assay for CT [20] has been optimized and a CE assay for the BC component of ACC has been developed.

Materials and methods

Reagents and assay solutions

Acetyl-CoA, malonyl-CoA, ATP, ADP, biocytin, dimethyl sulfoxide (DMSO), potassium phosphate monobasic, potassium bicarbonate, benzoic acid, magnesium chloride, and sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Andrimid [(2*E*,4*E*,6*E*)-*N*-((*S*)-3-(((*S*)-3-methyl-1-((3*R*,4*S*)-4-methyl-2,5-dioxopyrrolidin-3-yl)-1-oxobutan-2-yl)amino)-3-oxo-1-phenylpropyl)octa-2,4,6-trienamide] was a gift from Pfizer. 2-Amino-*N*,*N*-dibenzyloxazole-5-carboxamide was synthesized according to the protocol published by Mochalkin and coworkers [3]. Tris [tris(hydroxymethyl)aminomethane] was purchased from Acros Organics (Morris Plains, NJ, USA). Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) was purchased from EMD Millipore (Billerica, MA, USA). Sodium phosphate and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). BC and CT from *E. coli* were purified as described previously [21]. BCCP was a gift from Pfizer. All solutions were prepared using ultrapure water (>18 MΩ cm, ModuLab water system, United States Filter, Palm Desert, CA, USA). All buffers were filtered through a 0.2-μm nylon membrane (Whatman, Hillsboro, OR, USA) prior to use for capillary electrophoresis.

CE enzyme assays

All assays were performed using a P/ACE MDQ system with UV absorbance detection (photodiode array detector) from Beckman Coulter (Brea, CA, USA). The data were collected and analyzed using 32 Karat 5.0 software from Beckman Coulter. Fused-silica capillary (50 μm i.d. and 360 μm o.d.) was purchased from Polymicro Technologies (Phoenix, AZ, USA) and cut to a total length of 59.8 cm. The polyimide coating was removed using a MicroSolv CE window maker (Eatontown, NJ, USA) to make a 3-mm detection window at 49.8 cm (effective length). The capillary was rinsed with 1.0 M NaOH, ultrapure water, and 20.0 mM sodium phosphate buffer at pH 7.55 for 10.0 min at 20 psi for conditioning at the beginning of each day. All reaction vials were held at 25 °C, and the capillary was held at 25 °C. All injections were performed hydrodynamically at 0.5 psi for 10.0 s. All separations were performed using 30.0 kV (508 V/cm). All electropherograms were plotted with absorbance at 256 nm.

All enzyme assay reaction samples were prepared in 5.00 mM potassium phosphate buffer at pH 7.55 (reaction buffer). The capillary was rinsed with the separation buffer for 30.0 s at 20 psi prior to each injection. All enzyme reactions were performed off-column in a 1.5-ml glass Beckman CE vial in a 1.0-ml total volume.

CT CE assay

The assay mixture contained 250.0 μM malonyl-CoA and 4.00 mM biocytin in the reaction buffer. To initiate the reaction, CT was added to the reaction mixture to a final concentration of 10.0 μg/ml. The assay mixture was injected for CE analysis after 1.0 min and again after 9.5 min. The CT assay was carried out using 5.0 mM potassium phosphate buffer at pH 7.55, which was also used for the separation buffer.

BC CE assay

The BC assay mixture contained 50.0 μM ATP, 2.5 mM $MgCl_2$, 50.0 mM biotin, 5.0 mM potassium bicarbonate, and 200 μM benzoic acid (used as an internal standard) in the reaction buffer. To initiate the reaction, BC was added to the reaction mixture to a fi-