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A 96-well plate assay for high-throughput analysis of holocarboxylase synthetase activity

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Abstract

Background—Holocarboxylase synthetase (HCS) catalyzes the covalent binding of biotin to both carboxylases and histones. Biotinylated carboxylases and biotinylated histones play crucial roles in the metabolism of fatty acids, amino acids, and glucose, and in gene regulation and genome stability, respectively. HCS null mammals are not viable whereas HCS deficiency is linked to developmental delays in humans and phenotypes such as short life span and low stress resistance in *Drosophila*.

Methods—HCS-dependent biotinylation of the polypeptide p67 was detected and quantified in a 96-well plate format using IRDye-streptavidin and infrared spectroscopy.

Results—Biotinylation of p67 by recombinant HCS (rHCS) and HCS from human cell extracts depended on time, temperature, and substrate concentration, all consistent with enzyme catalysis rather than non-enzymatic biotinylation. The Michaelis-Menten constant of rHCS for p67 was $4.1 \pm 1.5 \mu\text{mol/l}$. The minimal concentration of rHCS that can be detected by this assay is less than 1.08 nmol/l . Jurkat cells contained 0.14 ± 0.02 units of HCS activity [μmol of biotinylated p67 formed/($\text{nmol/l HCS} \times \text{h}$)] in $400 \mu\text{g}$ of total protein.

Conclusions—We developed a 96-well plate assay for high-throughput analysis of HCS activity in biological samples and studies of synthetic and naturally occurring HCS inhibitors.

Keywords

assay; biotin; holocarboxylase synthetase; human; recombinant

1. Introduction

Holocarboxylase synthetase (HCS, E.C. 6.3.4.10) was identified by Suzuki et al. [1,2]; it has long been known that HCS catalyzes the covalent attachment of biotin to distinct lysine residues in carboxylases [3]. Mammalian cells express the following five carboxylases: acetyl-CoA carboxylase 1 in the cytoplasm, and acetyl-CoA carboxylase 2, 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase, and pyruvate carboxylase in mitochondria. Biotinylated carboxylases play essential roles in the metabolism of fatty acids, amino acids, and glucose [4,5].

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More recently, it was demonstrated that HCS also catalyzes the binding of biotin to lysine residues in histones [6]. Lysine residues in histones H3 and H4 are better targets for biotinylation than lysine residues in other histones [7–10], but some biotinylation sites have also been identified in histone H2A [11]. Biotinylation of histones plays essential roles in gene repression and genome stability [12–14].

Consistent with the role of HCS as essential protein biotinyl ligase, the monomeric enzyme is widely distributed throughout mammalian cells and has been detected in cytoplasm, mitochondria, and nuclei [1,15,16]. Three splicing variants of HCS have been identified in human placenta with molecular weights of 86, 82, and 76 kDa [15,17] but additional variants might exist [1]. Nuclear HCS is a chromatin protein [18] and appears to be enriched near the nuclear lamina [16].

To date, no living HCS *null* individual has been identified, suggesting embryonic lethality. HCS knockdown studies (~30% residual activity) produced phenotypes such as decreased life span and heat resistance [18] and increased frequency of retrotransposition events in *Drosophila melanogaster* [13]. Numerous mutations in the human *HCS* gene have been identified and characterized at both the enzymatic and clinical level; these mutations cause a substantial decrease in HCS activity [19,20]. Unless diagnosed and treated early, HCS deficiency appears to be uniformly fatal [21]. In most individuals clinical signs improve if treatment with pharmacological doses of biotin is initiated early [19–22]. In addition to mutations, about 2,200 single nucleotide polymorphisms have been mapped in the *HCS* locus but their importance for human health is unknown [23].

The prevention of adverse effects of HCS deficiency depends on the early diagnosis of enzyme activity. As of today, analysis of HCS activity depends on indirect assays that quantify the abundance of biotinylated carboxylases (“multiple carboxylase deficiency”). These assays are cumbersome and do not lend themselves to the analysis of a large number of samples, as required for studies of enzyme kinetics in HCS-deficient individuals. Moreover, our laboratory is interested in the identification of synthetic and natural HCS inhibitors that specifically target distinct subcellular structures. Such inhibitors would provide useful tools to distinguish biological effects mediated by biotinylation of cytoplasmic and mitochondrial carboxylases from those mediated by biotinylation of nuclear histones. Here we took an important step towards deciphering HCS biology and developed a 96-well assay for high-throughput analysis of both purified HCS and HCS in biological samples.

2. Materials and methods

2.1. Principle of assay

The assay is based on the HCS-dependent biotinylation of a polypeptide named p67, followed by detection and quantification of p67-bound biotin in a 96-well plate format using IRDye-streptavidin and infrared spectroscopy. p67 comprises the 67 C-terminal amino acids in human propionyl-CoA carboxylase, including the biotin-binding site lysine-669, and is a widely accepted substrate in studies of HCS [24]. Briefly, a mixture (50 μ l) of 75 mmol/l Tris-acetate (pH 8.0; all concentrations are final), 7.5 mmol/l ATP, 0.3 mmol/l dithiothreitol, 0.3 mmol/l biotin, 45 mmol/l $MgCl_2$, 6 nmol/l recombinant HCS (rHCS), and 8 μ mol/l recombinant p67 was incubated for 2 h at 37°C in microplates, unless noted otherwise (“Results”). Recombinant proteins were stored for up to 6 months without appreciable loss of activity (data not shown); dithiothreitol and ATP were stored in small aliquots at –20°C. Following the 2-h incubation, reactions were terminated by mixing 40 μ l of sample with 60 μ l of coating buffer containing 50 mmol/l sodium carbonate (pH 9.6) and 200 mmol/l sodium EDTA in black 96-well plates with clear bottoms (Corning, New York, NY; catalog

number 3601); plates were incubated overnight at 4°C on a rotating shaker to coat the plates with biotinylated p67. The coating buffer was removed, wells were washed twice with distilled water, and blocked with 200 µl of blocking solution {0.1% bovine serum albumin (wt./vol) and 0.05% Tween-20 (vol./vol) in phosphate-buffered saline (PBS)} for at least 4 h at 4°C. The blocking solution was discarded, and plates were washed twice with PBS and incubated with 100 µl of IRDye®-800CW-streptavidin for 1 h on a rotator at room temperature. IRDye-streptavidin was prepared by diluting a commercial stock solution (LI-COR, Lincoln, NE; catalog number 926-32230; 2.4 moles of IRDye 800 CW/mole of streptavidin, 1 mg/ml) 4,000-fold in 0.1% Tween 20 in PBS. Plates were washed four times with 0.1%-Tween-20 in PBS. Binding of IRDye-streptavidin to biotinylated p67 was quantified using the 800CW channel in an Odyssey infrared imaging system (LI-COR). HCS- or p67-free samples were used as negative controls.

2.2. Recombinant HCS (rHCS)

A clone of human HCS fused to glutathione S-transferase, S-tag, and both N-terminal and C-terminal 6x his-tags was expressed and purified as described [6] with the following modifications. Cells were collected by centrifugation at 4°C (3,000 × g for 30 min) and resuspended in 15 ml of PBS with protease inhibitor cocktail (Sigma, St. Louis, MO). Samples were sonicated on ice (Branson 250 Digital Sonifier, Danbury, CT) for 7 min with a 10-sec burst (30% amplitude) alternating with 20-s resting periods. Lysates were centrifuged at 10,000 g for 10 min at 4°C to remove debris. The supernatant was collected and rHCS fusion protein was purified by using GSTrap FF columns on an AKTA protein purification system (GE Health Care; Piscataway, NJ). Protein purity was confirmed by gel electrophoresis and staining with coomassie blue; bioactivity of rHCS was tested using gel electrophoresis with recombinant p67 as substrate as described [6]. p67- and HCS-free samples were used as negative controls. Protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL).

2.3. Expression and purification of p67

Recombinant p67 was used as substrate for HCS activity assays. His-tagged p67 was purified using His Trap FF columns on an AKTA protein purification system as described [10]; purity was confirmed by using gel electrophoresis and staining with coomassie blue. The microbial HCS ortholog BirA is known to biotinylate an appreciable fraction of recombinant p67 from *E. coli* [10]; hence, the biotinylated fraction of recombinant p67 was removed using avidin columns as described [10]. Protein concentrations were determined using the bicinchoninic acid method.

2.4. Assay calibration

Assays were calibrated by preparing dilution series of IRDye-streptavidin in PBS in black 96-well plates. One molecule of streptavidin can bind up to four molecules of biotin [25], but the true extent of biotin binding in this plate assay is unknown. Hence, the assay was calibrated by assuming that steric hindrance allowed for the binding of only 2 molecules of biotin were per molecule of streptavidin. Infrared absorbance of IRDye-streptavidin was measured at 800 nm in the Odyssey imaging system. Non-linear regression analysis was used to calculate K_m and V_{max} of HCS for p67 (GraphPad Prism 5.00; La Jolla, CA).

2.5. Analysis of HCS in human cells

Whole cell extracts from 150×10^6 Jurkat human lymphoid cells were prepared using 500 µl of cold lysis buffer (5 mmol/l PIPES, pH 8.0, 85 mmol/l KCl, 0.5% NP40) in the presence of protease inhibitors [26]. Cells were sonicated on ice (Branson 250 Digital Sonifier, Danbury, CT) with a 5-s burst (30% amplitude). Complete lysis was confirmed by visual

inspection under a light microscope. Debris was removed by centrifugation at 200 g at 4°C for 5 min; the supernatant contained 17.3 µg/µl total protein. Activity of endogenous HCS was quantified by incubating 23 µl of supernatant (~400 µg protein) with recombinant p67 and cofactors in a final volume of 50 µl for 2 h at 37°C in the plate assay. Negative controls were prepared by omitting supernatant and by heating supernatants for 2 min at 99°C.

2.6. Statistical analysis

Bartlett's test was used to confirm that variances were homogeneous [27]. Significance of differences among more than two groups was tested by one-way ANOVA and Fisher's Protected Least Significant Difference procedure for post hoc testing. Student's paired *t*-test was used for pairwise comparisons. StatView 5.0.1 (SAS Institute, Cary NC) was used to perform all calculations. Differences were considered statistically significant if $P < 0.05$. Data are expressed as mean $\pm$ S.D.

3. Results

3.1. Recombinant HCS and p67

Purified rHCS produced a protein of the expected size (114.6 kDa due to tags) and was >90% pure as judged by staining with coomassie blue (Fig. 1A, lane 1). Likewise, his-tagged recombinant p67 produced a chemically pure protein of the expected size, judged by staining with coomassie blue (Fig. 1A, lane 2). When rHCS was incubated with p67 and cofactors, a strong biotinylation signal was detected in p67 using streptavidin as a probe (Fig. 1A, lane 3), consistent with the notion that the rHCS produced in this study is bioactive. Importantly, we confirmed our previous observation that recombinant p67 is biotinylated by BirA in *E. coli* (Fig. 1B, lane 1) and therefore requires affinity purification before use. When recombinant p67 was purified using avidin columns, the vast majority of biotinylated p67 was removed (Fig. 1B, lane 2). Biotin-depleted p67 was used for all assays described below.

3.2. Temperature and time

The catalytic activity of rHCS depended on temperature and time. When various amounts of rHCS were incubated with p67 and cofactors at either 4°C or 37°C, enzyme activities were significantly higher at 37°C (Fig. 2); representative examples (2.2 nmol/l and 5.8 nmol/l rHCS) are depicted. At 4°C, enzyme activities were slightly above background noise. This is consistent with enzyme-mediated rather than non-enzymatic biotinylation of p67. All subsequent experiments were conducted at 37°C.

When various amounts of rHCS were incubated with p67 and cofactors for 1, 2, or 3 h, enzyme activities increased linearly for up to 2 h, but leveled off thereafter. For example, enzyme activities (7.2 nmol/l rHCS) were 12.6 ± 2.2 of infrared absorbance after 1 h, 28.8 ± 2.9 after 2 h, and 34.1 ± 2.2 after 3 h (Fig. 3). Values are expressed in arbitrary units of infrared absorbance (800 nm) until assay optimization and calibration was achieved (see below). All subsequent experiments were conducted using 2-h incubations.

3.3. Concentrations of HCS and p67

The amount of p67 that was enzymatically biotinylated increased linearly up to a concentration of rHCS of 8 nmol/l, and leveled off thereafter as indicated by the transition from a solid to a dashed line (Fig. 4). Subsequent experiments were conducted using 6 nmol/l HCS to ensure assay linearity. Furthermore, an rHCS dilution series was conducted to determine the apparent detection limit of rHCS in the plate assay. At a concentration of 1.08 nmol/l HCS, biotinylation of p67 was significantly greater than in negative controls without

rHCS, whereas 0.54 nmol/l HCS were not significantly different from background controls ($n = 3$, $P < 0.05$; data not shown).

Dose-response studies were conducted with varying amounts of p67 to confirm that substrate availability was not a limiting factor in this plate assay. A linear dose-response was obtained for up to 5.2 $\mu\text{mol/l}$ p67, while at higher concentrations of p67, the overall reaction rate exhibited saturation as indicated by the transition from a dashed to a solid line (Fig. 5). These findings were further corroborated by enzyme kinetics studies. The apparent K_m of rHCS for p67 was $4.1 \pm 1.5 \mu\text{mol/l}$ and the apparent V_{max} was 34.9 ± 4.1 units of infrared absorbance (Fig. 6). For subsequent experiments, p67 was used at a concentration of 8 $\mu\text{mol/l}$.

3.4. Assay calibration

A dilution series of IRDye-streptavidin was prepared to calibrate the HCS assay. One molecule of streptavidin can bind up to four biotin molecules [25]; we assumed that each molecule of IRDye-streptavidin bound, on average, two molecules of biotin under the conditions of this assay. The dilution series of IRDye-streptavidin produced a linear response across all concentrations tested (Fig. 7), and the assay system was re-calibrated daily. A typical sample of rHCS under the conditions described in the preceding sections produced a signal of about 36 units of infrared absorbance, suggesting that 0.16 μmol of p67 were biotinylated when incubated with 6 nmol/l HCS for 2 h at 37°C. We define one unit of HCS activity as 0.08 μmol of biotinylated p67 formed $\times \text{nmol/l}^{-1} \text{HCS} \times \text{h}^{-1}$ at 37°C.

3.5. HCS activity in Jurkat cells

Supernatants from Jurkat cells contained 0.14 ± 0.02 units of HCS activity in 400 μg of total protein (0.02 ± 0.03 units of HCS activity per 10^6 cells); no signal was detected in cell-free controls and heat-treated protein extracts (negative controls).

4. Discussion

Here, we present a 96-well plate assay for high-throughput screening of HCS activity based on the detection of biotinylated p67 using IRDye-streptavidin and infrared absorbance. Previous assays of HCS activity are based on the biotinylation of p67 in a gel electrophoresis-based format. While we acknowledge that the gel-based assay is slightly more sensitive than the plate assay (not shown), we also note that the plate assay has the advantages that large numbers of samples (including replicates) can be analyzed rapidly and cost-efficiently. Despite its lower sensitivity, analysis of HCS in cell extracts by plate assay is feasible, as evidenced by successful quantification of enzyme activity in Jurkat cell extracts. Moreover, the plate assay does not require the use of radiochemicals, unlike some of the other assays of HCS deficiencies in patients with multiple carboxylase deficiency [19]. We envision two possible applications for the plate assay. First, the assay can be used in the analysis of enzyme kinetics for mutant or variant human HCS, including newborn screening. Second, the assay could be particularly useful when screening libraries of synthetic or naturally occurring compounds for potential HCS inhibitors in a high-throughput format. With the newly discovered HCS-dependent biotinylation of histones and the role of histone biotinylation in gene regulation and genome stability [12–14,28], the need for robust HCS assays is likely to increase.

This study confirmed our previous observations with regard to endogenous biotinylation of recombinant p67. Recombinant p67 contains biotin and requires affinity purification prior to use in HCS assays [10]; otherwise, artifactual overestimate of HCS activity will occur. The yield of bioactive rHCS is low, if produced following protocols published in the 1990s, at

least when we attempted to use these protocols in our laboratory. We suspect that the low yield of bioactive rHCS by previous protocols is caused by improper folding of the protein; hence, we devised a protocol tailored towards maximizing the yield of rHCS [6].

One obstacle to the broad use of this 96-well plate assay is the requirement for an infrared spectrophotometer. If no such instrumentation is available, we speculate that the assay can be modified by substituting streptavidin-peroxidase for IRDye-streptavidin, thereby allowing the use of a standard plate optical spectrophotometer quantitating absorption settings of 450 nm. We conclude that a robust method was developed for a robust assay of HCS activities in both clinical and research settings.

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Abbreviations

HCS	holocarboxylase synthetase
K_m	Michaelis-Menten constant
PBS	phosphate-buffered saline
rHCS	recombinant HCS
V_{max}	maximal velocity

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Fig 1. Recombinant HCS and p67. (A) Recombinant HCS (lane 1) and p67 (lane 2) were purified using affinity chromatography and stained with coomassie blue (CB); p67 was incubated with rHCS and co-factors, and biotin was probed with streptavidin (SA, lane 3). (B) Biotin in recombinant p67 was probed with streptavidin before (lane 1) and after (lane 2) purification of p67 by avidin chromatography.

Fig. 2.

Temperature dependency of p67 biotinylation. Various amounts of rHCS were incubated with p67 and cofactors at 4°C and 37°C; enzyme activities were measured by infrared spectroscopy. Bars without a common letter are significantly different when comparing the same concentrations of rHCS ($n = 3$, $P < 0.05$).

Fig. 3.

Effect of incubation time on rHCS activity. rHCS was incubated with p67 and cofactors for 1, 2 or 3 h at 37°C; enzyme activity was measured by infrared spectroscopy. Bars without a common letter are significantly different ($n = 3$, $P < 0.05$)

Fig. 4. Biotinylation of p67 depends on the concentration of rHCS. Recombinant p67 was incubated with various concentrations of rHCS at 37°C for 2 h, while all other variables were kept constant. Biotinylation of p67 was quantified using infrared absorbance with IRDye-streptavidin as a probe for biotin.

Fig. 5.

Determination of optimal p67 concentrations. Recombinant HCS (6 nmol/l) was incubated with various concentrations of p67 at 37°C for 2 h, while all other variables were kept constant. Biotinylation of p67 was quantified using infrared absorbance with IRDye-streptavidin as a probe for biotin.

Fig 6. Kinetics of p67 biotinylation by rHCS. The line was fitted to the Michaelis-Menten equation by nonlinear regression analysis ($n = 3$).

Fig. 7.

Assay calibration. A standard curve was produced by quantifying the infrared absorbance of IRDye-streptavidin (up to 0.8 nmol/well) at 800 nm. Note that 0.8 nmol/well IRDye-streptavidin were considered to be the equivalent of 1.6 nmol/well biotin (n = 3).