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A Colorimetric Assay Method to Measure Acetyl-CoA Synthetase Activity: Application to Woodchuck Model of Hepatitis Virus-induced Hepatocellular Carcinoma

Yu Kuang¹, Nicolas Salem¹, Fangjing Wang¹, Steve J Schomisch^{2,*}, Visvanathan Chandramouli^{3,*}, and Zhenghong Lee^{4,1,♦}

¹Department of Biomedical Engineering, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA

²Division of Cardiothoracic Surgery, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, Ohio 44106, USA

³Department of Medicine, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106, USA

⁴Department of Radiology, University Hospitals of Cleveland, 11100 Euclid Avenue, Cleveland, OH 44106, USA

Abstract

A new spectrophotometric method for quantitation of acetyl-CoA synthetase (ACAS) activity is developed. It has been applied for ACAS assay in the liver tissues of a woodchuck model of hepatitis virus-induced hepatocellular carcinoma (HCC). The assay is based on the established pyrophosphate (PPi) detection system. ACAS activity is indexed by the amount of PPi, the product of ACAS reaction system of activated form of acetate (acetyl-CoA) with ACAS catalysis. PPi is determined quantitatively as the amount of chromophore formed with molybdate reagent, 1-amino-2-naphthol-4-sulfonic acid in bisulfite and 2-mercaptoethanol. PPi reacts with molybdate reagent to produce phosphomolybdate and PPi-molybdate complexes. 2-mercaptoethanol is responsible for color formation which has the peak absorption at 580nm. This method was sensitive from 1 to 20 nmol of PPi in a 380- μ l sample (1-cm cuvette). A ten-fold excess of Pi did not interfere with the determination of PPi. To study the major metabolic pathways of imaging tracer [1-¹¹C]-acetate in tumors for detection of HCC by Positron Emission Tomography (PET), the activity of one of the key enzymes involved in acetate or [1-¹¹C]-acetate metabolism, ACAS was assayed by this newly developed assay in the tissue samples of woodchuck HCCs. An significant increase of ACAS activity was observed in the liver tissues of woodchuck HCCs as compared with neighboring regions surrounding the tumors ($P < 0.01$). The respective ACAS activities in the subcellular locations were also significantly higher in HCCs than in the surrounding tissues ($P < 0.01$) (total soluble fraction: 876.61 ± 34.64 vs. 361.62 ± 49.97 mU/g tissue; cytoplasmic fraction: 1122.02 ± 112.39 vs. 732.32 ± 84.44 mU/g tissue; organelle content: 815.79 ± 491.5 vs. 547.91 ± 97.05 mU/g tissue; sedimentable fragment: 251.92 ± 143.7 vs. 90.94 ± 18.98 mU/g tissue). The finding suggests an increase in ACAS activity in the liver cancer of woodchuck models of HCC as compared to that in the normal woodchuck liver. The

♦Corresponding Author: Zhenghong Lee, Ph.D. Nuclear Medicine, Radiology, University Hospitals of Cleveland 11100 Euclid Avenue, Cleveland, OH 44106, USA Tel: (216)844-7920 Fax: (216)844-3106 E-mail: ZXL11@case.edu

*These authors contributed equally to this work.

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developed assay is rapid, simple and accurate and is suitable for the investigation of ACAS activity under physiologic and pathophysiologic conditions.

Keywords

Acetyl-CoA Synthetase; Woodchuck; Woodchuck Hepatitis Virus; Hepatocellular Carcinoma; [1-¹¹C]Acetate; Positron Emission Tomography

1. Introduction

Acetyl CoA synthetase (E.C. 6.2.1.1; ACAS), a ubiquitous enzyme found in microrganisms, mammalian tissue and higher plant, catalyzes the following reaction: acetate + ATP + CoA → acetyl-CoA + ADP + PPi. Synthesis of the activated form of acetate (acetyl-CoA) is carried out by the enzyme acetyl-CoA synthetase utilizing acetate, CoA, and ATP and generating PPi as the byproduct of the reaction. ACAS is present in the liver cells both in cytosol and mitochondria. Because of the catalysis of ACAS, the intravenously injected radiotracer for Positron Emission Tomography (PET) imaging, [1-¹¹C]-acetate showed high uptake in the liver tumor of woodchuck (*Marmota monax*) model of hepatocellular carcinoma (HCC) and showed much lower uptake and slower clearance of the tracer in non-neoplastic woodchuck liver tissues. In addition, during our PET imaging studies on the same woodchuck model of HCC, [1-¹¹C]-acetate was rapidly taken up in the myocardium and subsequently metabolized to CO₂ via the tricarboxylic acid cycle. The preferred metabolic pathway(s) of [1-¹¹C]-acetate in primary liver cancer such as HCC is not clearly understood. After converting into acetyl-CoA by acetyl-CoA synthetase, [1-¹¹C]-acetate might enter a synthesis route, most likely for lipid synthesis, which is different from the metabolic pathway within myocardium. We hope that acetate imaging will facilitate early detection of HCC. To study the exact metabolism of [1-¹¹C]-acetate in liver tumors, the activity of the first enzyme involved, ACAS needs to be assayed.

Most of the currently available methods that can measure ACAS are based on either acetyl-CoA or PPi formation by coupling acetyl-CoA or PPi to other enzyme systems. Stern et al. [1] coupled the acetyl-CoA with acetylating enzyme. Aas et al. [2] coupled the acetyl-CoA with carnitine acetyltransferase. A more sensitive method for coupling the PPi formation with other enzyme systems is to convert the PPi into Orthophosphate (Pi) and then couple with 2-amino-6-capto-7-methylpurine ribonucleoside in a three-step reaction system [3]. These methods suffer from a potential non-enzymatic hydrolysis problem due to endogenous biological orthophosphate-containing compound from tissue extracts which interferes with results under ultraviolet or visible light. On the other hand, the main limitation of enzymatic-coupled assay system lies in difficulties to accommodate the different special optimal reaction conditions for each individual enzyme within one reaction assay mixture. Huang [4] developed a sensitive, rapid, radiochemical assay method for ACAS which measured the direct incorporation of ¹⁴C-acetate into acetyl-CoA. This radiometric determination method has good sensitivity but is inherently incontinent due to the use of long half life of radio isotope ¹⁴C. A novel colorimetric assay has been developed in this study that can directly measure PPi produced by acetate with ACAS catalysis.

This paper reports the development of a sensitive, rapid and simple colorimetric assay method to measure ACAS activity by direct determination of PPi as a product of the synthesis reaction system of activated form of acetate (acetyl-CoA) with ACAS catalysis. This enzymatic assay method is based on established well-characterized pyrophosphate detection system originally described by Putnins et al [5]. In principle, as the product of ACAS reaction system, PPi is determined quantitatively as the amount of chromophore formed with molybdate reagent, 1-

amino-2-naphthol-4-sulfonic acid in bisulfite and 2-mercaptoethanol. PPi reacts with molybdate reagent to produce phosphomolybdate and PPi-molybdate complexes. 2-mercaptoethanol is responsible for color formation which has the peak absorption at 580nm [5,6]. The method was sensitive up to 20 nmol of PPi in a 380- μ l sample (1-cm cuvette) and colorimetry was performed at 580nm as indicated. In addition, a ten-fold excess of Pi does not interfere with the determination of PPi in this method. In a mixed PPi and Pi system, PPi forms a colored molybdate complex. Pi and molybdate also react in strong sulfuric acid to form 12-molybdophosphoric acid which is then reduced to molybdenum blue by the reducing agents. These two complexes can be differentiated by the use of two reducing agents, Eikonogen and 2-mercaptoethanol, and by measuring the absorbance at 580 nm for PPi in the absorption spectra of the two chromophores [5,6]. The method was then applied to measurement of ACAS level in the liver tissue of woodchuck HCC and provided the quantitative evidence for the first key step of [1-¹¹C]-acetate metabolism in HCC, which is helpful for the understanding of the preferred metabolic pathway(s) of [1-¹¹C]-acetate in HCC.

2. Materials and Methods

2.1. Reagents

Acetyl-CoA synthetase, CoA, dithiothreitol (DTT), ATP, Trizma base, ammonium molybdate, sodium pyrophosphate, sodium phosphate dibasic, thioglycolic acid, 2-mercaptoethanol and 1-amino-2-naphthol-4-sulfonic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium bisulfite was purchased from Fisher scientific Inc. (Hampton, NH).

2.2. Preparation of biological samples for analysis

Thawed woodchuck liver tissue samples were homogenized in pre-cooled homogenization buffer (pH 7.4) (1 g / 3 ml) containing 10 mM Tris-HCl, 250mM sucrose and 1 mM 2-mercaptoethanol. Four different subcellular fractions of liver tissues were obtained [7,8]. First, resected tissues were homogenized and sonicated (three pulses at 15 seconds, 20KHz) at 4°C to disrupt cellular membranes (Branson Sonifier 450, VWR Scientific). After centrifugation for 30 minutes at 30,000 $\times$ g (Beckman L7 ultracentrifuge, Ti90 rotor; Beckman Coulter, Inc.), the supernatant was collected. This portion was called the total soluble fraction. Other tissues were directly centrifuged after homogenization for 30 minutes at 30,000 $\times$ g before the supernatant was collected. This portion was called the cytoplasmic fraction. The remaining pellet was subjected to three 15-seconds pulses of ultrasound before centrifugation at 30,000 $\times$ g for 30 minutes. The supernatant, representing the organelle content (nuclei, mitochondria and microsomes), was collected, and the remaining pellet contained all sedimentable fragments. Aliquots (1.0ml each) were stored at -80°C for assaying their ACAS activity.

2.3. Acetyl-CoA synthetase assay

For ACAS reaction, the following reagents were combined in a 1-ml reaction volume: 50mM Tris-HCl (pH 7.8), 10 mM sodium acetate, 4 mM ATP, 0.15 mM CoA, 1 mM magnesium chloride, 10mM DTT and acetyl-CoA synthetase containing sample. The control reaction contained all of the components of the reaction, except acetyl-CoA synthetase. Following incubation at 37 °C for 20 min, the reaction was terminated and 380 μ l of supernatant was transferred to a test tube. 50 μ l of 2.5% molybdate reagent, 50 μ l of 0.5 M 2-mercaptoethanol and 20 μ l of Eikonogen was added for a total volume of 0.5 ml. At equilibrating for 10 min, the absorbance at 580 nm was determined using a Beckman DU spectrophotometer (Beckman DCI[®] 520 general purpose UV/VIS). The background was prepared by similar treatment of the samples except that the samples were denatured before added. The amount of PPi attributed to the action of acetyl-CoA synthetase was determined by subtracting the background absorbance at 580 nm measured in the control reaction with the absence of enzyme. The total protein content in the ACAS sample was also determined by the method of Bradford (Bio-Rad

Laboratories, Inc) [9]. The activity was expressed in units per ml of sample and then normalized to the total protein content.

2.4. PPI and Pi standard calibration curves

The standard PPI solution contained 1.0 mM $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ was prepared fresh and diluted before use. The standard Pi solution contained 1.0mM anhydrous Na_2HPO_4 was stored at 2°C and diluted before use. 2.5% ammonium molybdate was prepared via dissolving in 5N H_2SO_4 (without orthophosphate admixtures). Eikonogen reagent was prepared as follows: 0.25g of sodium sulfite, 14.65 g of meta-bisulfite, and 0.25 g of 1-amino-2-naphthol-4-sulfonic acid were dissolved in 80 ml of hot distilled water. The solution was cooled to 25°C, filtered and adjusted to a final volume of 100 ml [5]. The reagent was stored in an amber bottle at room temperature for 2 months. 1-amino-2-naphthol-4-sulfonic acid should be a roseate color. It becomes darker during long storage and thus has to be recrystallized by dissolving in hot water in the presence of sulfite and meta-bisulfite with subsequent precipitation at cold temperatures by HCl [10]. The preparation thus obtained was stored in darkness. 0.2~0.4 unit/ml of ACAS was prepared by adding ACAS into appropriate volumes of distilled, deionized H_2O . The ACAS solution was immediately divided into convenient aliquots, stored at -20°C and thawed just before use.

Increasing amount of 0.1 mM $\text{Na}_4\text{P}_2\text{O}_7$ or NaH_2PO_4 were added with 50 μl of 2.5% molybdate reagent, 50 μl of 0.5 M 2-mercaptoethanol and 20 μl of Eikonogen in a total volume of 0.5 ml. Following incubation at 37°C, the absorbance at 580 nm was measured within 10~60 min in a Beckman DU spectrophotometer (Beckman DCI® 520 general purpose UV/VIS). The background absorbance in the absence of $\text{Na}_4\text{P}_2\text{O}_7$ or NaH_2PO_4 was subtracted, and the net change in absorbance was plotted to construct a standard curve. To test the key role of 2-mercaptoethanol in the color formation of spectrophotometric measurement, an additional standard mixture was also prepared as above but without 2-mercaptoethanol.

2.5. Optimization for enzymatic reaction conditions

The optimal pH of the reaction buffer was examined between 6.5 and 8.5 for 10mM 10 mM Tris-HCl buffer. The linearity of the enzymatic reaction was determined by varying the incubation time (10-30 min).

2.6. Application of the assay

Woodchucks bearing HCC induced by woodchuck hepatitis virus (WHV) and woodchucks with normal liver (mass 2.5 – 5 kg) were obtained from Cornell University (courtesy of Dr. Bud Tennant). The animal protocol followed IACUC approved procedures, and experiments were conducted in accordance with the guidelines of the NIH “Guide for the Care and Use of Animals”. After dynamic PET imaging (the imaging study is reported elsewhere), each of the woodchucks was sacrificed and the liver was immediately harvested from the animal. A pathologist separated HCC portion from the surrounding hepatic liver. Specimens were snap frozen in liquid nitrogen and stored at -80°C for later enzyme activity assays. Both HCC specimens and surrounding hepatic liver specimens from infected woodchucks were processed for ACAS activity assays. The normal liver specimens from healthy woodchuck were used as control. The procedure of ACAS assay was performed as described as above.

2.7. Statistical Analysis

Comparisons of ACAS activities between the HCC region and normal region in the liver of the same HCC woodchuck and between the HCC tissues and normal woodchuck liver tissues were performed by one way analysis of variance (ANOVA) or ANOVA on ranks when

appropriate. The level of significance was set at $p < 0.05$. Statistical analysis was performed using SigmaStat® (SPSS Inc., Chicago, IL, USA).

2.8. Definition of Unit and Specific Activity

One unit of ACAS activity is defined as 1 μ mole acetyl-CoA formed per minute at pH 7.5 and 37°C. Specific activity of ACAS is expressed as units per milligram of protein. In order to compare the enzyme activity distribution of sub-cellular locations within the same tissue sample, the activity was also express as units per mg of tissue.

3. Results

3.1. PPi and Pi Standard calibration curve

The amount of PPi generated in the ACAS assay can be determined by constructing a standard curve. The standard curves were constructed using NaH_2PO_4 as the source of Pi and $\text{Na}_4\text{P}_2\text{O}_7$ as the source of PPi (Fig 1). The linear relationship between the concentration of PPi and absorbance was observed up to 20 nmole PPi or Pi per tube with the presence of 2-mercaptoethanol. Comparison of the two curves showed that each molecule of PPi has quantitatively 3-fold higher absorbance than each molecule of Pi. This higher absorbance of PPi enhances the sensitivity of the assay for PPi at 580 nm which is linear from 1 to approximately 20 nmoles in the 1-ml reaction volume. Previous results demonstrated that 2-mercaptoethanol was found to be the most effective accelerator of the reaction as previously indicated [5], which is obligatory for color formation contributed to the maximal absorption spectra at 580 nm for PPi. This is consistent with what we observed from the standard curve of PPi and Pi with the 2-mercaptoethanol omitted as shown in Fig 1. With the addition of 25 μ moles of 2-mercaptoethanol into the standard mixture, the absorbance was maximal after 10 min and was stable up to at least 60 min. There were less detectable colors within PPi and Pi standard reaction mixture at 580 nm in the absence of 2-mercaptoethanol.

3.2. Mixed PPi and Pi samples

The pyrophosphate detection system used in this ACAS assay has a high specificity for PPi in the presence of a 10-fold excess of orthophosphate as previously indicated [5]. Fig 2 shows a standard calibration curve of a mixed sample which had PPi with 7 nmoles Pi added. The curve was similar with the normal standard calibration curve and linear with PPi concentration. The absorbance also increased 3-fold when 2-mercaptoethanol was added as compared with the absence of 2-mercaptoethanol. The absorbance of mixed PPi and Pi samples was additive with 2-mercaptoethanol present as shown in Fig 2. PPi could be determined in such mixed systems by measuring the absorbance of Pi with the absence of 2-mercaptoethanol, converting this to the absorbance of Pi with the presence of 2-mercaptoethanol via a standard curve. The PPi amount can then be determined by subtracting this value from the Pi + PPi absorbance with the presence of 2-mercaptoethanol [5,6].

3.3. Dependence of acetyl-CoA synthetase activity on time

In this ACAS assay, absorbance is initially linear with respect to time with a fixed amount of ACAS used. As shown in Fig 3, the assay worked in a linear fashion following the incubation at 37°C. The absorbance increased linearly with time and up to 30min.

3.4. Effect of pH on acetyl-CoA synthetase activity in Tris buffer

The effect of pH on acetyl-CoA synthetase activity in Tris buffer was illustrated in Fig 4. Our result indicated that the optimal pH of acetyl-CoA synthetase catalyzes reaction is 7.8 in Tris buffer.

3.5. Comparison of ACAS activity between normal woodchuck liver tissues and HCC tissues and between HCC region and surrounding region within the liver of the same HCC woodchuck

The activities of ACAS in HCC tissues, surrounding hepatic tissues and in normal woodchuck liver are reported in Figure 6. A higher ACAS activity was found in WHC-induced HCCs than in surrounding hepatic regions. Fig. 5 shows all pairwise multiple comparison procedures using Tukey test. We noted that the normalized ACAS activity in woodchuck HCCs were significantly higher than those in surrounding regions ($P < 0.01$). The absolute ACAS activities was also significantly higher in HCCs than in the surrounding tissues ($P < 0.01$) (total soluble fraction: 876.61 ± 34.64 vs. 361.62 ± 49.97 mU/g tissue; cytoplasmic fraction: 1122.02 ± 112.39 vs. 732.32 ± 84.44 mU/g tissue; organelle content: 815.79 ± 491.5 vs. 547.91 ± 97.05 mU/ g tissue; sedimentable fragment: 251.92 ± 143.7 vs. 90.94 ± 18.98 mU/ g tissue).

4. Discussion

We propose the method described above for the assay of ACAS activity by the use of two reducing agents, Eikonogen and thiol reagent. ACAS activity is indexed by the amount of PPi, the product of ACAS reaction system of activated form of acetate (acetyl-CoA) with ACAS catalysis. The PPi forms a colored molybdate complex that is reduced and develops a table color sufficiently fast. It is obvious that the alternation of PPi amount which reflects the activity of ACAS can be determined by this method.

To study the major metabolic pathways of imaging tracer $[1-^{11}\text{C}]$ -acetate in tumors for detection of HCC by PET, the activity of ACAS, which is one of the key enzymes involved in acetate or $[1-^{11}\text{C}]$ -acetate metabolism, was assayed by this newly developed assay in the tissue samples of woodchuck HCCs. An significant increase of ACAS activity was observed in the liver tissues of woodchuck HCCs as compared with neighboring regions surrounding the tumors ($P < 0.01$) (Fig 5).

As mentioned above, the usefulness of $[1-^{11}\text{C}]$ -acetate PET imaging for HCC depends on the mechanism(s) of $[1-^{11}\text{C}]$ -acetate upatke in HCCs, which is (are) still unclear. The degree of cell differentiation was demonstrated to affect the amount of tracer accumulation in hepatocytes. In the dual isotope 2- $[^{18}\text{F}]$ Fluoro-2-deoxy-D-glucose (FDG) / $[1-^{11}\text{C}]$ -acetate PET study of Ho et al. [11], the poorly differentiated HCCs were detected by FDG and the well-differentiated types were detected by $[1-^{11}\text{C}]$ -acetate. In addition, in those liver tumors that are both FDG and $[1-^{11}\text{C}]$ -acetate positive (the moderately differentiated type), some cases suggested that these tracers are taken up by different parts of the tumor. Therefore, it is indicated that mixed metabolic constituents and kinetics are likely to be present within the same tumor, but at variable degrees.

ACAS catalyzes the ligation of acetate with CoA to produce acetyl-CoA, which is involved in various metabolic pathways such as fatty acid synthesis, cholesterol synthesis, ketogenesis and the tricarboxylic acid cycle etc. As the first step for studying the metabolic fate of acetate in neoplasms, we measured the ACAS activity on liver samples of woodchuck models of HCC using the method developed here. Acetate utilization has been demonstrated in many tissues with different metabolic pathways in different species [12-14]. Subcellular fractionation revealed that the hepatic-type enzyme of ACAS (termed ACAS1) is a cytosolic enzyme, whereas the cardiac-type enzyme (termed ACAS2) is located in the mitochondrial matrix [15]. The ACAS1 provides acetyl-CoA for the synthesis of fatty acids and cholesterol; the ACAS2 provide acetyl-CoA that is utilized mainly for oxidation under ketogenic condition [15,16] and it also contributes to the activation of acetate incorporats into fatty acids or cholesterol [17]. Studies have shown that the main localization of ACAS in hepatocytes varies among species. Quraishi et al. [18] reported that most of the ACAS activity was located in the

mitochondria in bovine liver. In contrast, Ballard et al. [14] reported that a significant fraction of ACAS activity in sheep liver was found in the cytoplasm. Two types of ACAS are found in rat hepatocytes: one located in the cytosol, and one located in the mitochondrial matrix, which is different with the ACAS in heart mitochondria [19]. Luong et al. [16] suggested that the hepatic type ACAS was mainly a cytosolic enzyme in human. It is obvious from our study that the ACAS activity is highest located predominantly in cytosolic fraction (cytosol and microsomes) in woodchuck liver tissue samples from the absolute enzyme activity data (mU/g tissue) (Figure 6). A similar finding was presented for rat liver tissues [14] and sheep liver tissues [15]. The differences of ACAS activities among different subcellular fractions reflected the site of metabolism of acetate: acetate carbon entering the malonyl-CoA pathway and lipid synthesis in the liver. Contrary to that, in the heart, the major function of ACAS is believed to be in the tricarboxylic acid cycle as previously reported due to the evidence that over 80% of ACAS activity was associated with the mitochondrial fraction [12,14,20].

The functional activity of ACAS within woodchuck liver depends upon the intracellular concentrations of substrates and the affinity of the enzymes for those substrates. In the fed animals the availability of acetate would favor the synthetase-catalyzed reaction with ACAS catalysis, whereas during starvation an increase in fatty acid oxidation would produce excess acetyl CoA and the formation of acetate would be favored [21]. In our study, however, there was a significantly higher ACAS activity in tumor regions of woodchuck liver tissues than in the surrounding regions of woodchuck liver tissues following overnight fasting before dynamic PET scans. This may suggest the apparent excess of ACAS activity and enhanced lipid synthesis present in woodchuck liver tissue where neoplasm is formed. This observation leads to the hypothesis that enhanced ACAS in HCC tissue would contribute to [1-¹¹C]-acetate incorporating preferentially into the lipids via forming acetyl-CoA, which serves as a substrate for fatty acid synthesis and triglyceride formation, rather than into the amino acids or CO₂ during the PET scan procedure. Yoshimoto et al. [21] suggested that phospholipids (especially phosphatidylcholine) and neutral lipids are the main components of the lipid fraction that labeled acetate will end up in tumor cells. Phosphatidylcholine is synthesized mainly from acetate through the CDP-choline pathway with choline kinase catalysis, whose activity increases when the cells are neoplastically transformed [22]. Additional analysis is needed to further confirm preferential incorporation of [1-¹¹C]-acetate into phosphatidylcholine in the tumor liver tissue of woodchuck model of HCC.

In both normal and malignant cells, three major pathways—lactate dehydrogenase, the respiratory chain, and lipid synthesis—are involved in the regulation of the redox balance. Interestingly, in some cancer cells, because intracellular O₂ supplies decline (i.e., hypoxic condition), the respiratory chain cannot fully oxidize the reducing equivalents being formed and, in this metabolic context, increased lipogenesis has been proposed to be the main physiologic function to maintain redox balance through oxidation of NADPH during fatty acid synthesis [23]. Our findings about the increased ACC activity in woodchuck HCC in this study will help in gaining understanding about the mechanism of [1-¹¹C]-acetate retention in primary human liver cancer.

In conclusion, a new method has been developed for assay ACAS activity. As opposed to other spectrophotometric assays for ACAS activity, this method does not require multiple steps and tedious procedures requiring several buffers and reagents. It also avoids the use of long half life radionuclide, which is suitable for the studies of the ACAS activity under physiological and pathophysiological conditions. Using this method, we have found a significant difference in ACAS activity between HCCs and surrounding hepatic tissues in woodchucks.

5. Simplified description of the method and its applications

The method proposed in this paper is based on the amount of PPI released by the ACAS reaction system of activated form of acetate (acetyl-CoA) with ACAS catalysis, which is indexed as ACAS activity. PPI is determined quantitatively as the amount of chromophore formed with molybdate reagent, 1-amino-2-naphthol-4-sulfonic acid in bisulfite and 2-mercaptoethanol. PPI reacts with molybdate reagent to produce phosphomolybdate and PPI-molybdate complexes. 2-mercaptoethanol is responsible for color formation which has the peak absorption at 580nm. A ten-fold excess of Pi did not interfere with the determination of PPI. The reported colorimetric assay for ACAS activity of Woodchuck Model of HCC shown in this paper has significant advantages over current enzyme coupled system assays that introduce new variables, for example the origin of the coupled enzyme and endogenous biological orthophosphate-containing compound from tissue extracts which interferes with results under ultraviolet or visible light. It is more accurate, simpler, reliable, cheap and avoids the use of radiochemical assays.

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Abbreviations

ACAS, Acetyl-CoA Synthetase; FDG, [^{18}F]-2-fluoro-2-deoxy-D-glucose; HCC, Hepatocellular Carcinoma; PET, Positron Emission Tomography; PPI, Pyrophosphate; Pi, Orthophosphate; WHV, Woodchuck hepatitis virus.

Reference

1. Stern JR, Shapiro B, Stadtman ER, Ochoa SJ. Enzymatic synthesis of citric acid. III. Reversibility and mechanism. *J Biol Chem* 1951;193:703–720. [PubMed: 14907760]
2. Aas M, Brenner J. Short-chain fatty acid activation in rat liver. A new assay procedure for the enzymes and studies on their intracellular localization. *Biochim Biophys Acta* 1968;164:157–166. [PubMed: 5721019]
3. Upson RH, Haugland RP, Malekzadeh MN, Haugland RP. A spectrophotometric method to measure enzymatic activity in reactions that generate inorganic pyrophosphate. *Anal Biochem* 1996;243:41–45. [PubMed: 8954523]
4. Huang KP. A sensitive assay method of acetyl CoA synthetase. *Anal Biochem* 1970;37:98–104. [PubMed: 5506569]
5. Putnins RF, Yamada EW. Colorimetric determination of inorganic pyrophosphate by a manual or automated method. *Anal Biochem* 1975;68:185–195. [PubMed: 171970]
6. Manusurova SE, Deryabina OA. A simple colorimetric assay method for pyrophosphate in the presence of a 1000-fold excess of orthophosphate: application of the method to the study of pyrophosphate metabolism in mitochondria. *Anal Biochem* 1989;176:390–394. [PubMed: 2545112]
7. Gouillat C, Manganas D, Zoulim F, Vitrey D, Saquier G, Guillaud M, Ain JF, Duque-Campos R, Jamard C, Praves M, Trepo C. Woodchuck hepatitis virus-induced carcinoma as a relevant natural model for therapy of human hepatoma. *J Hepatol* 1997;26:1324–1330. [PubMed: 9210620]
8. Ash R, Baird GD. Activation of volatile fatty acids in bovine liver and rumen epithelium. *Biochem J* 1973;136:311–319. [PubMed: 4359516]
9. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:238–254.

10. Manusurova SE, Deryabina OA. A simple colorimetric assay method for pyrophosphate in the presence of a 1000-fold excess of orthophosphate: application of the method to the study of pyrophosphate metabolism in mitochondria. *Anal Biochem* 1989;176:390–394. [PubMed: 2545112]
11. O'Brien ML, Spear BT, Glauert HP. Role of oxidative stress in peroxisome proliferator-mediated carcinogenesis. *Crit Rev Toxicol* 2005;35:61–88. [PubMed: 15742903]
12. Woodnutt G, Parker DS. Rabbit liver acetyl-CoA synthetase. *Biochem J* 1978;175:757–759. [PubMed: 33661]
13. Hanson RW, Ballard FJ. The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. *Biochem J* 1967;105:529–536. [PubMed: 5583995]
14. Ballard FJ. Supply and utilization of acetate in mammals. *AM J Clin Nutr* 1972;25:773–779. [PubMed: 4558368]
15. Fujino T, Kondo J, Ishikawa M, Morikawa K, Yamamoto TT. Acetyl-CoA synthetase 2, a mitochondrial matrix enzyme involved in the oxidation of acetate. *J Bio Chem* 2001;276:11420–11426. [PubMed: 11150295]
16. Luong A, Hannah VC, Brown M, Goldstein JL. Molecular characterization of human acetyl-CoA synthetase, an enzyme regulated by sterol regulatory element-binding proteins. *J Bio Chem* 2000;275:26458–26466. [PubMed: 10843999]
17. Goldberg RP, Brunengraber H. Contributions of cytosolic and mitochondrial acetyl-CoA synthetases to the activation of lipogenic acetate in rat liver. *Adv Exp Med Biol* 1980;132:413–418. [PubMed: 6106997]
18. Quraishi S, Cook RM. Utilization of volatile fatty acids in ruminants. IV. Relative activities of acetyl CoA synthetase and acetyl CoA hydrolase in mitochondria and intracellular localization of acetyl CoA synthetase. *J Agr Food Chem* 1972;20:91–95. [PubMed: 5059948]
19. Yamashita H, Fukuura A, Makamura T, Kaneyuki T, Kimoto M, Hiemori M, Tsuji H. Purification and partial characterization of acetyl-coA synthetase in rat liver mitochondria. *J Nutr Sci Vitaminol (Tokyo)* 2002;48:359–364. [PubMed: 12656208]
20. Knowles SE, Jarrett IG, Filsell OH, Ballard FJ. Production and utilisation of acetate in mammals. *Biochem J* 1997;142:401–411. [PubMed: 4441381]
21. Yoshimoto M, Waki A, Yonekura Y, Sadato N, Murata T, Omata N, Takahashi N, Welch MJ, Fujibayashi Y. Characterization of acetate metabolism in tumor cells in relation to cell proliferation: acetate metabolism in tumor cells. *Nucl Med Biol* 2001;28:117–122. [PubMed: 11295421]
22. Macara IG. Elevated phosphocholine concentration in ras-transformed NIH 3T3 cells arises from increased choline kinase activity, not from phosphatidylcholine breakdown. *Mol Cell Biol* 1989;265:6042–6047.
23. Hatzivassiliou G, Zhao F, Bauer DE, Andreadis C, Shaw AN, Dhanak D, Hingorani SR, Tuveson DA, Thompson CB. ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer Cell* 2005;8:311–321. [PubMed: 16226706]

Fig 1.

Standard calibration curve of PPi and Pi. Absorbance at 580 nm versus nmoles of PPi and Pi with 2-mercaptoethanol (2-ME) present or absent. The standard reaction mixture containing different amounts of 0.1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 μl of 2.5% molybdate reagent and 20 μl of Eikonogen in a final volume of 0.5 ml with 25 μmoles of 2-mercaptoethanol or without 2-mercaptoethanol was used. Following incubation at 37°C, the absorbance at 580 nm was spectrophotometrically measured within 10~60 min. The data represent the average of the same standard reaction mixture performed in triplicate.

Figure 2.

Mixed PPI and Pi assay. Fig. 1. Standard calibration curve for mixed PPI + 7 nmoles Pi systems. Absorbance at 580 nm versus nmoles of PPI and Pi with 2-mercaptoethanol (2-ME) present or absent. The standard reaction mixture containing different amount of 0.1 mM $\text{Na}_4\text{P}_2\text{O}_7$ plus 7 nmoles Pi, 50 μl of 2.5% molybdate reagent and 20 μl of Eikonogen in a final volume of 0.5 ml with 25 μmoles of 2-mercaptoethanol or without 2-mercaptoethanol was used. Following incubation at 37°C, the absorbance at 580 nm was spectrophotometrically measured within 10~60 min. The data represent the average of the same standard reaction mixture performed in triplicate.

Figure 3.

Dependence of ACAS activity on time. The experiment was carried out by withdrawing 380 μ l reaction mixture from reaction mixture of ACAS assay at each 5 min interval. The increase in absorbance at 580 nm was recorded over time.

Figure 4.
Effect of pH on acetyl-CoA synthetase activity in Tris buffer.

The Specific Activities of Acetyl-CoA Synthetase on Woodchuck Model of HCC (unit: mU/mg protein)

*P<0.05

Figure 5.
Acetyl-CoA Synthetase activities in the woodchuck livers

The Subcellular Distribution of Acetyl-CoA Synthetase on Woodchuck Model of HCC (Unit: mU/ g tissue)

*P<0.05

Figure 6.
The Distribution of Acetyl-CoA Synthetase activities in the subcellular location of woodchuck livers