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Cyclohexanedione Herbicides are Inhibitors of Rat Heart Acetyl-CoA Carboxylase

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Abstract—Acetyl CoA carboxylase (ACC) catalyzes the carboxylation of acetyl CoA to form malonyl CoA. In skeletal muscle and heart, malonyl CoA functions to regulate lipid oxidation by inhibition of carnitine palmitoyltransferase-1, an enzyme which controls the entry of long chain fatty acids into mitochondria. We have found that several members of the cyclohexanedione class of herbicides are competitive inhibitors of rat heart ACC. These compounds constitute valuable reagents for drug development and the study of ACCβ, a validated anti-obesity target.

Introduction

Malonyl CoA, the product of acetyl CoA carboxylase (ACC), is an important regulator of lipid metabolism in wide range of plant and animal species.[1–3] In mammalian heart and skeletal muscle, which rely primarily on free fatty acids for energy, malonyl CoA produced by ACCβ binds to a regulatory site on carnitine palmitoyltransferase **1** (CPT-1) and inhibits the enzyme, which regulates the entry of fatty acids into mitochondria for β-oxidation.[2–4] Malonyl CoA produced by ACCα serves an anabolic role as a substrate for de novo lipid synthesis catalyzed in lipogenic tissues by fatty acid synthase.[5] At the cellular level, pools of malonyl CoA generated by the different ACC isoforms appear to function separately, perhaps due to the different subcellular location of the enzymes.[6] ACCβ and CPT-1 are in close proximity to one another on the mitochondrial outer membranes, whereas ACCα and FAS are in the cytosol.[5,7]

ACC works through a two-step reaction mechanism, with the first half-reaction being the ATP-dependent carboxylation of covalently bound biotin.[5,8] In the second carboxyltransferase reaction, the substrate acetyl CoA is carboxylated to form malonyl CoA.[5] Citrate is a potent allosteric activator of both isoforms of ACC, and serine phosphorylation by AMPK serves as a primary means of negative regulation.[9,10]

With the elucidation of ACCβ's role in regulation of lipid metabolism, it has become recognized as a potential point for therapeutic intervention in metabolic diseases such as diabetes and obesity.[3,6,11] The recent publication of the ACCβ−/− mouse phenotype has further validated the enzyme as a drug target. These mice are fertile and have a normal life span, yet they have substantially reduced fat mass and increased rates of lipid oxidation in skeletal muscle.[6]

In order to establish tools to study mammalian ACCβ, we have characterized several members of the cyclohexanedione (CHD) class of herbicides, which act by inhibiting ACC in sensitive monocotyledonous plants.[1] In contrast to previous reports that CHDs do not inhibit mammalian ACC,[12–14] our results show that several of the cyclohexanedione herbicides are inhibitors of mammalian ACC and therefore constitute useful reagents for the study of this enzyme.

Results and Discussion

Rat ACC was partially purified from heart, where ACCβ is the predominant isoform.[15] Analysis of the enzyme preparation by SDS-PAGE and coomassie staining showed a major band at ~285k Da, the predicted weight for ACCβ. A lower, less intensely stained

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band at the predicted weight for ACCα (260 kDa), was visible with longer times of electrophoresis (Fig. 1B). Quantification of these bands showed ACCβ was 64% of total and ACCα 36%, similar to protein levels reported for ACC isoforms isolated from rat skeletal muscle.[16] Western blotting with a polyclonal antibody to the unique N-terminus of ACCβ showed a strong signal from a single band on the gel (Fig. 1A, lane 2). An antibody to a phospho-peptide present in both ACCα and β also reacted strongly with a single band in the preparation.

Several representative CHDs were chosen to determine whether this class of compound would inhibit rat heart ACC (Fig. 2). ACC activity was measured in vitro by the carboxylation of acetyl-CoA with [^{14}C]-HCO_3^- to form [^{14}C]-malonyl CoA. Clethodim, cycloxydim, sethoxydim, tepraloxydim and tralkoxydim were all found to inhibit rat heart ACC with similar potency (IC_{50} values between 18 and 40 μM, Fig. 3). Alloxydim was much less active (IC_{50} >100 μM, Fig. 3).

To determine the mechanism of CHD inhibition, kinetic studies were carried out with the representative compound clethodim. Varying concentrations of inhibitor versus substrates (Fig. 4, A–C) or the allosteric activator citrate (D) show that clethodim inhibition is most sensitive to increasing concentrations of acetyl CoA. A double reciprocal plot of the data (Fig. 4A, inset) suggests that clethodim is competitive with respect to acetyl CoA. Kinetic analysis with cycloxydim, sethoxydim, tepraloxydim and tralkoxydim demonstrated that these compounds were also competitive with acetyl CoA (not shown).

Decreased utilization of lipid is predictive for, and associated with, obesity in humans and rodents.[11,18–20] ACCβ plays a pivotal role in regulating the balance between lipid and carbohydrate utilization, and in recent years it has emerged as a well-validated anti-obesity target. ACCβ−/− mice have increased rates of lipid oxidation in muscle and a large decrease in fat mass and body weight.[6] Based on these and other findings, there is a compelling case for the development of a small molecule inhibitor of human ACCβ.

One current problem with ACCβ drug development efforts is the paucity of well-characterized, small molecule inhibitors for in vitro assay development. Some inhibitors that have been previously described are active

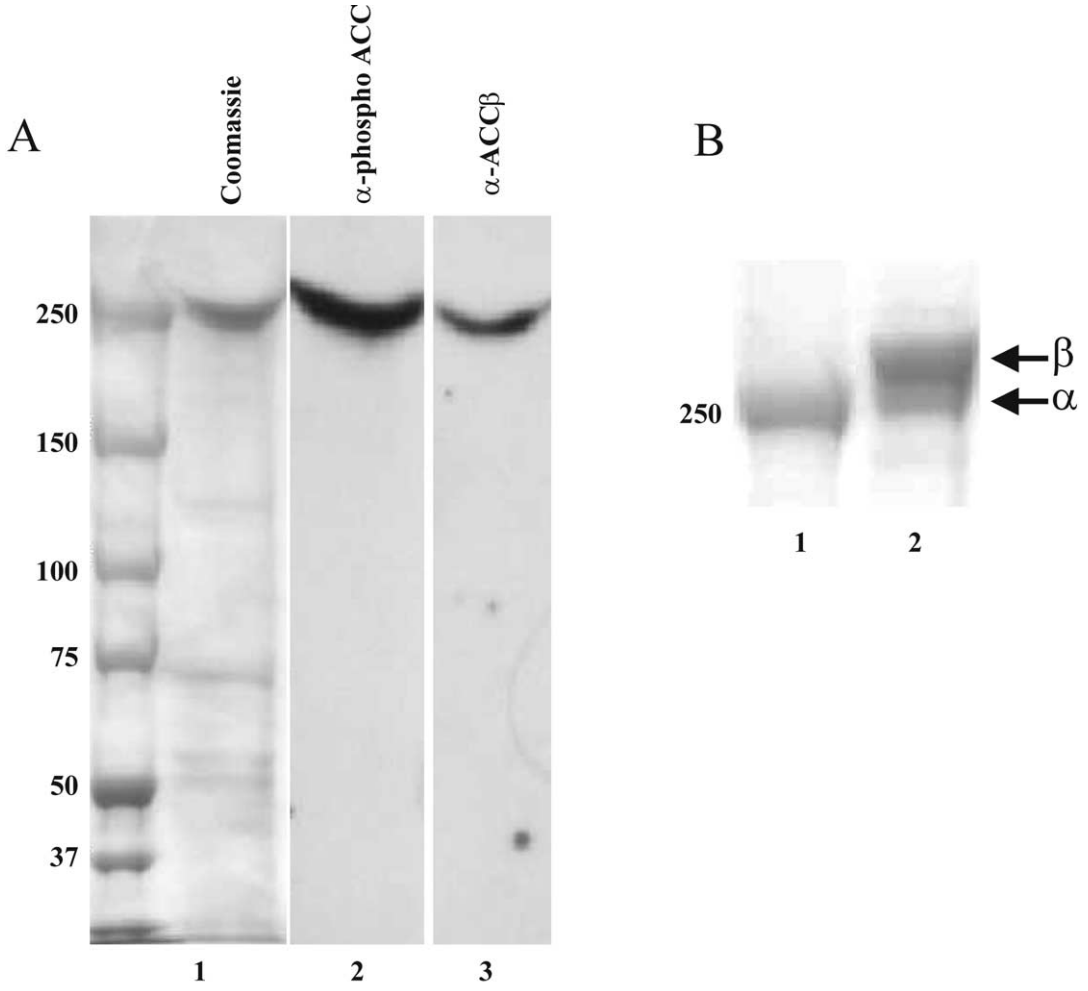

Figure 1. Purification of ACC from rat heart tissue. (A) Purified ACC was concentrated and analyzed by 3–8% tris acetate SDS-PAGE and coomassie staining (lane 1). The enzyme was also detected by immunoblotting with antibodies to either phosphorylated ACC (lane 2) or the unique N-terminal sequence of ACCβ (lane 3; (B) longer times of electrophoresis resolved α and β isoforms.

only as coenzyme A thioesters and are therefore not as useful for establishing cell-free screens.[21,22] Other small molecule inhibitors such as clofibrate and nafenopin have known activity at other targets and low potency at mammalian ACC.[23]

In this study we have demonstrated that several cyclohexandione herbicides are inhibitors of rat heart acetyl CoA carboxylase in vitro. We have also shown that these inhibitors are competitive with the substrate acetyl-CoA. This is consistent with previous studies that have mapped the site of CHD inhibition of wheat ACC to the carboxyltransferase domain, where the inhibitors are nearly competitive with acetyl-CoA.[14,24] In the carboxyltransferase domain, residues 1763-2333 of rat ACCβ, the amino acid identity between rat ACCβ and CHD sensitive plants such as corn and wheat is ~50%. The precise roles of cyclohexanedione core functional groups and substituents at R_{1-5} (Fig. 2A) in binding mammalian ACC are not known, but mapping studies with corn ACC suggest that interaction is favored by bulk groups and low negative charge in the oxime/R_4 region. These studies also show that bulk groups contribute positively to ACC binding in the R_1 region and negatively in R_2.[25]

It is not clear why we have found inhibition of the rat heart ACC by CHDs while others have reported no inhibition of mammalian ACC with sethoxodim.[12] The rat ACCβ lacks an isoleucine residue in the carboxyltransferase domain that has been associated with sensitivity to CHDs,[13] but it is clearly sensitive to many of these compounds (Figs. 3 and 4). Enzyme preparations from tissues other than heart (e.g., liver, adipose), which were used in previous studies, may be somewhat less sensitive due to the higher proportion of expressed ACCα.[12,15,16] However, preliminary data from our group show enzyme from these sources is also inhibited by clethodim. We think the most likely reason for the discrepancy is differences in enzyme preparation and assay conditions between studies, as we find these parameters can substantially impact the observed potency of

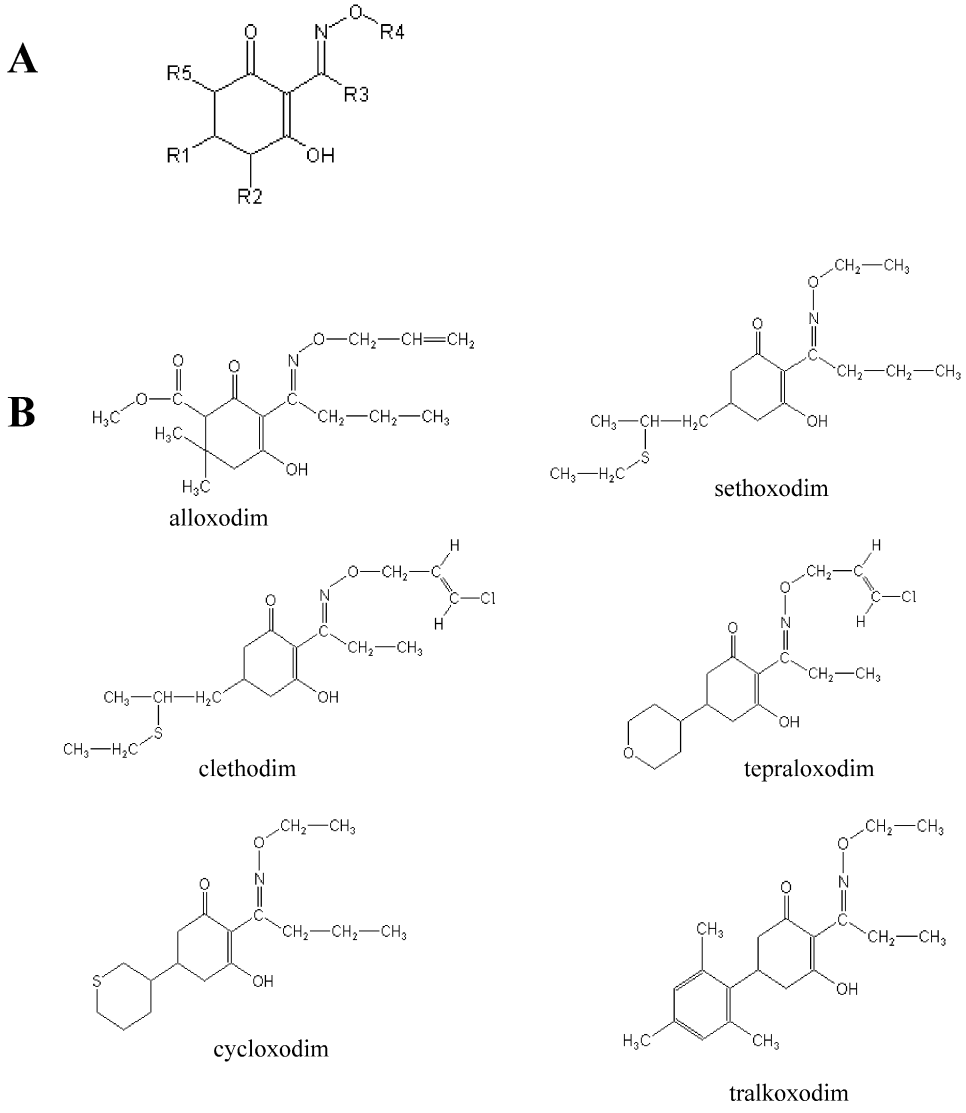

Figure 2. (A) Core structure of active cyclohexanedione (CHD) herbicides; (B) representative CHDs.

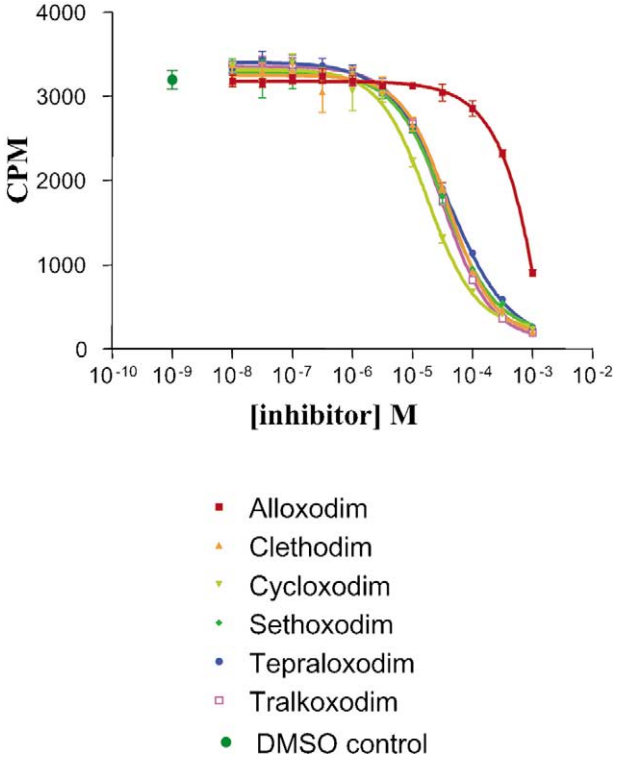

Figure 3. Inhibition of rat heart ACC by cyclohexanedione herbicides. ACC activity is monitored by the incorporation of [^{14}C]-HCO_3^- into malonyl CoA. Inhibitor concentration is plotted versus CPM of acid stable [^{14}C]-malonyl CoA produced. Data shown are derived from two independent experiments, each run in duplicate. Error bars represent standard error of the mean.

ACC inhibitors.

In summary, we have identified several members of the cyclohexanedione family of herbicides that inhibit rat heart acetyl CoA carboxylase. These inhibitors are competitive with the substrate acetyl CoA and are not sensitive to concentrations of ATP, bicarbonate or citrate. It is anticipated that these inhibitors will prove useful for the study of ACC and the development of therapeutic inhibitors.

Experimental

Materials

Cycloxydim, trepraloxydim, and alloxydim were purchased from Dr. Ehrenstorger, GmbH, Augsburg, Germany. Sethoxydim, tralkoxydim and clethodim were from Chem Service, West Chester, PA, USA. All other reagents were from Sigma, Saint Louis, MO, USA, unless otherwise indicated.

Purification of ACC

ACC was purified essentially as outlined by Trumble, Smith and Winder.[16] Briefly, 100 rat hearts (Pel-Freez, Rogers, Ark.) were homogenized in a Waring blender for 2 min in 500 mL of Buffer A (225 mM mannitol, 75 mM sucrose, 10 mM Tris/HCl pH 7.5, 5 mM potassium citrate, 2.5 mM $MnCl_2$, 0.05 mM EDTA, and Roche complete protease inhibitor and the homogenate was then centrifuged at 4 °C for 30 min at 17,000*g*. The supernatant was brought to 35% ammonium sulfate and stirred for 45 min at 4 °C, then centrifuged again for 30 min at 17,000*g*. The pellet was re-suspended in buffer B (100 mM Tris/HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 10% glycerol) and centrifuged at 40,000*g* for 20 min at 4 °C to remove insoluble material. The supernatant was then dialyzed against 4 L buffer C (100 mM Tris/HCl, 500 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT and 5% glycerol) for 3 h at 4 °C using 100,000 molecular weight cut-off dialyis tubing. Biotinylated protein in the supernatant was bound to a an avidin affinity column (Softlink, Promega) and eluted with 5 mM biotin and stored at −80 °C.

ACC activity assay

The carboxylase assay was essentially that of Thampy and Wakil,[17] with modifications. The assay buffer contained 100 mM HEPES pH 7.5, 20 mM $MgCl_2$, 20 mM potassium citrate, 2 mM DTT, 4 mM ATP and 1.5% fish gelatin. The assay was performed in a 96-well polypropylene plate and initiated by adding 100 μL of assay buffer containing approximately 30 pg of purified ACC to 40 μL of water containing 2.0 μCi [^{14}C]-bicarbonate (NEC086H) and 41.7 μM acetyl CoA, for a final reaction volume of 140 μL. Reactions were allowed to proceed for 1.5 h at room temperature and were then terminated by the addition of 50 μL 1 N HCl. Acid was mixed with the reaction by pipeting, and 140 μL was removed to a 96-well Millipore GFB filter plate. Samples were dried overnight in a desiccator, 30 μL of Optiphase liquid scintillant was added to each well, and plates were counted in a Wallac beta counter. The assay used for determining bicarbonate competition with clethodim will be published elsewhere.

Antibodies

An ACCβ-specific polyclonal antibody was raised in rabbits to the peptide CVPSKEDKKQANIKR, which was coupled to keyhole limpet hemocyanin. Antibody production and affinity purification was performed by Zymed laboratories, San Francisco, CA, USA. Anti-phospho-ACC rabbit polyclonal antibody was purchased from Upstate Biotechnology, Lake Placid, NY, USA.

Electrophoresis and immunoblotting

Purified ACC was concentrated using Millipore Mirocon 30,000 MWCO concentrators and run on NuPAGE 3–8% Tris–acetate gel. For immunoblots, proteins were transferred to Millipore Immobilon P, incubated with 1 μg/mL primary antibodies in 1% bovine serum albumin in PBS for 1 h at room temperature. Blots were then washed for 15 min with multiple changes of PBS and incubated with a 1:20,000 dilution of peroxidase conjugated goat anti-rabbit secondary

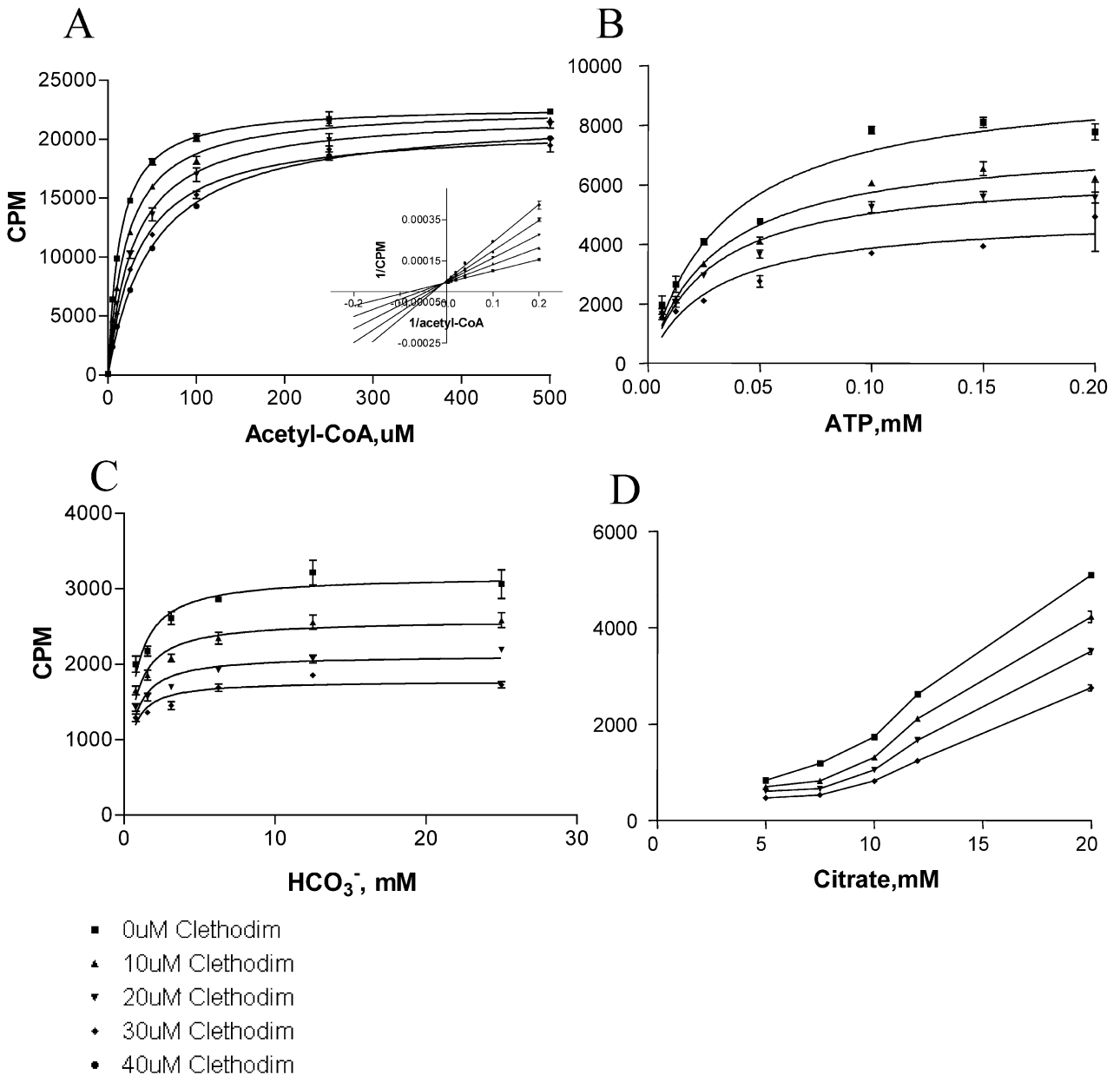

Figure 4. Kinetic analysis of rat heart ACC inhibition by clethodim. ACC activity was measured in the presence of 0, 10, 20, 30, or 40 mM clethodim at varying concentrations of the substrates (A) acetyl CoA, (B) ATP, (C) bicarbonate and the activator (D) citrate. The inset (A) shows the data for acetyl CoA in a double reciprocal Lineweaver–Burk plot. The graph in A is representative of four independent experiments run in duplicate. The data in B–D are representative of two independent experiments run in duplicate. Error bars represent standard error of the mean.

antibody (Sigma) in PBS/BSA for 1 h at room temperature. Following this, the blots were washed for 30 min with multiple changes of PBS and developed with Pierce Super Signal western blot developing agent, according to the manufacturers instructions. Quantification of coomassie stained gels was performed using Bio-Rad Quantity One software and a Bio-Rad Fluor-S imager.

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