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Effect of Oxaloacetate and Phosphorylation on ATP-Citrate Lyase Activity†

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ABSTRACT: ATP-citrate lyase (CL) catalyzes the conversion of citrate and CoA to oxaloacetate (OA) and acetyl-CoA. As the coupled malic dehydrogenase (MDH) assay is not able either to study the effect of oxaloacetate (OA) on CL activity or to measure accurately CL activity in biological samples, a new assay was developed. The CL−citrate coupled CAT assay measures the amount of acetyl-CoA formed by transferring radiolabeled acetyl-CoA synthesized from [^{14}C]citrate to chloramphenicol with chloramphenicol acetyltransferase (CAT). Employing this assay, the rate of increase in acetyl-CoA synthesis from citrate is linear with respect to added CL. Kinetic values for ATP, CoA and citrate are similar to those obtained using the MDH assay. The effect of CL phosphorylation on enzyme activity was determined. CL phosphorylated by cAMP-dependent protein kinase or by this kinase and glycogen synthase kinase-3 (GSK-3) decreases the apparent V_{max} without changing the apparent K_m. The effect of OA, a product of the enzyme reaction, on CL activity was also determined. Computational analysis of the data obtained without added OA and at three concentrations of OA indicate that the apparent K_m for the substrate is not altered even though the apparent V_{max} is decreased. The effect of OA on the activity of phosphorylated enzyme was also determined. OA decreases the apparent V_{max} of the phosphorylated enzyme to the same extent as in control CL. This assay is able to measure CL activity in cytosol from 3T3-L1 adipocytes. The activity in cytosol was inhibited by OA at lower concentrations than found for purified enzyme: K_i, 63 μM for CL activity in cytosol and 1.2 mM for purified CL. These results suggest that CL activity is affected both by OA and by phosphorylation.

Fatty acid synthesis depends on cytosolic acetyl-CoA that is derived from mitochondrial pyruvate dehydrogenase. Acetyl-CoA, unable to pass from mitochondria to cytosol, condenses with oxaloacetate (OA)[1] to form citrate which is transported to the cytosol. ATP-citrate lyase (CL)[1] (EC

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[1] Abbreviations: ACC, acetyl-CoA carboxylase; CAT, chloramphenicol acetyltransferase; CL, ATP-citrate lyase; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GSK-3, glycogen synthase kinase-3; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, isobutylmethylxanthine; MDH, malic dehydrogenase; OA, oxaloacetate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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4.3.1.8), a tetramer with a molecular weight about 440 000 (Singh et al., 1976), catalyzes the conversion of citrate and CoA to acetyl-CoA and OA. This pathway generates both acetyl-CoA for lipid synthesis (Pilkis & El-Maghrabi, 1988) and OA for possible gluconeogenesis in liver and α-glycerol phosphate synthesis in fat cells, via formation of phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (Sasaki et al., 1984).

CL activity varies with changing hormonal and metabolic states (Singh et al., 1976; Gibson et al., 1972; Mackall et al., 1976; Sul et al., 1984). These activity changes, which take hours to occur, are due to changes in amounts of enzyme (Elshourbagy et al., 1990) which is regulated at transcription by the effects of nutrients and hormones (Kim et al., 1992). In addition to CL being regulatory in long-term control of lipid synthesis, it also could be important in its short-term regulation, along with acetyl-CoA carboxylase (ACC)[1] (EC 6.4.1.2) activity which is regulated by both allosteric effectors and phosphorylation (Hardie, 1989).

ACC is phosphorylated by both cAMP-dependent and cAMP-independent protein kinases (Munday et al., 1988; Haystead & Hardie, 1988; Haystead et al., 1988; Witters et al., 1988) with its activity increasing with decreasing phosphorylation (Witters et al., 1988). CL is also phosphorylated by both cAMP-dependent and cAMP-independent protein kinases (Pierce et al., 1981; Ramakrishna & Benjamin, 1985; Price et al., 1989). No functional role has been assigned to CL phosphorylations even though in fat and liver cells nearly all CL undergoes phosphorylation changes that are complex (Benjamin et al., 1994).

CL has three phosphorylation sites, each four amino acids apart (threonine 446 and serines 450 and 454) (Ramakrishna et al., 1990). Serine 454 is phosphorylated by cAMP-dependent protein kinase and an insulin-stimulated kinase (Price et al., 1989; Yu et al., 1993). Threonine 446 and serine 450 are phosphorylated only by GSK-3[1] (Ramakrishna et al., 1990). The sequence about these phosphorylation sites strongly resembles glycogen synthase, sites 3_{a-d} (Price et al., 1989). Their phosphorylations, like those of glycogen synthase, are hormonally regulated (Woodgett & Cohen, 1984; Cohen, 1986; Ramakrishna et al., 1990).

β-Adrenergic agents increase phosphorylation of all three CL phosphorylation sites whereas insulin increases phosphorylation at serine 454 but decreases phosphorylation at threonine 446 and serine 450 (Ramakrishna et al., 1984, 1989, 1990). Insulin increases phosphorylation of serine 454 presumably by both increasing the activity of an insulin-stimulated kinase (Sutherland et al., 1993) and decreasing phosphorylation at sites 446 and 450 (Ramakrishna et al., 1984, 1990). This result in turn decreases GSK-3 activity (Ramakrishna & Benjamin, 1988; Hughes et al., 1992), making serine 454 a better substrate for further phosphorylation (Ramakrishna et al., 1989). This formulation is supported by Welsh and Proud (1993), who found that insulin decreases GSK-3 activity in CHO cells. Because we found that hormone- and differentiation-induced CL phosphorylation changes are substantial and GSK-3β content falls with differentiation in 3T3-L1 cells (Benjamin et al., 1994), a new study of CL was made.

The MDH assay which measures CL activity (Takeda et al., 1969) cannot study the effect of OA, a product of citrate cleavage, on the enzyme reaction nor is it able to assess CL activity accurately in unprocessed cell extracts, as in the first case OA is converted to malate by MDH and in the latter case the oxidation of NADH is not tightly coupled to citrate cleavage. In addition, as partial purification of CL from cell extracts may result in loss of regulatory factors and/or changes in its phosphorylation or primary structure, a new radiochemical assay for CL activity was developed. Using the CL−citrate coupled CAT assay, we found that OA is an inhibitor of enzyme activity. We also observed that *in vitro* CL phosphorylation by cAMP-dependent protein kinase or this kinase plus GSK-3 decreases the apparent V_{max} whereas the apparent K_m for citrate is unchanged.

EXPERIMENTAL PROCEDURES

Materials. CL was purified from rat liver (Ramakrishna & Benjamin, 1985; Wells, 1991). Rabbit polyclonal antibodies against purified rat CL holoenzyme are the same antibodies used in prior immunoprecipitation and Western blotting studies (Ramakrishna & Benjamin, 1985; Benjamin et al., 1994). The catalytic subunit of cAMP-dependent protein kinase was purified from rabbit skeletal muscle (Bechtel et al., 1977). GSK-3 was purified from rat liver (Ramakrishna et al., 1985; Hughes et al., 1992). Tissue culture media, fetal calf sera, bovine sera, Tween-20, insulin, dexamethasone, NADH,[1] CAT,[1] OA,[1] IBMX,[1] and chloramphenicol were from Sigma Chemical Corp. Acrylamide, as a 40% solution, was from B.D.H., England, and bis-(acrylamide), as a 2% solution, was from National Diagnostics Corp. Reagents for Western blotting were from Bio-Rad Corp. ECL[1] reagents were from Amersham. Ecolume was purchased from ICN Radiochemicals. Citric acid, [1,5-^{14}C], and chloramphenicol, D-*threo*-[1,2-^{14}C], were from New England Nuclear. Other chemicals were of the highest grade available. Protein was measured using the Bradford-Coomassie R250 dye technique (Bio-Rad).

New Assay for CL−Citrate Coupled CAT Method. CL activity was measured using the coupled MDH assay at pH 8.7 or at pH 7.5 (which gives about 40% of the activity measured at pH 8.7). Our new CL activity assay is based on transfer of acetyl-CoA synthesized from citrate to chloramphenicol by added CAT (Gorman et al., 1982). The acetylated radiolabeled forms of chloramphenicol are separated from the aqueous phase containing citrate by partition into ethyl acetate (organic phase) and assayed for radioactivity (Gorman et al., 1982). The assay, total volume of 100 μL, contains 50 mM HEPES,[1] pH 7.5, or 50 mM Tris·HCl, pH 8.7; DTT[1] at various concentrations; 10 mM $MgSO_4$; 0.66 mM CoA; 0.1−6 mM Mg·citrate with added [^{14}C]citrate, 0.1 μCi; 2.4 or 2.5 mM Mg·ATP; 6 units of CAT; and 1.2 mM chloramphenicol. Unless otherwise described, CL is activated by incubation with 10 mM DTT or 10 mM 2-mercaptoethanol for 20 min at 37 °C (Wells, 1991). CL (0.5−2.5 μg) is added to a complete reaction mixture (without ATP or citrate), and the assay is started by addition of ATP or citrate. Incubation is at 37 °C for varying times, most often 10 min. The reaction is stopped by heating the sample rapidly to 65 °C. Samples are cooled, and 20 μL of 100 mM Tris·HCl, pH 8.7, is added to each tube to assure that the pH is alkaline; 300 μL of ice-cold ethyl acetate is added and the mixture centrifuged at 14000*g* for 3 min; 270 μL of the organic (upper) phase is transferred to a fresh tube. Again, 300 μL of ice-cold ethyl acetate is added and centrifuged, and 300 μL of the organic phase is transferred to the extract. This step is repeated, giving a total of 870

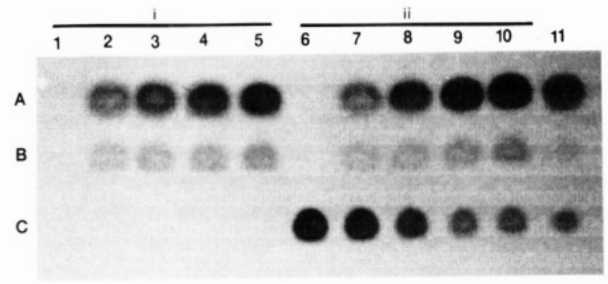

FIGURE 1: Products of CL (CL−citrate coupled CAT assay): Autoradiogram of products resolved by TLC. CL was incubated in a reaction mixture containing (final concentrations) Mg•[^{14}C]-citrate, 0.5 mM, and chloramphenicol, 1.2 mM, for samples in lanes 1−5 or Mg•citrate, 4.9 mM, and [^{14}C]chloramphenicol, 0.3 mM, for samples in lanes 7−10 or without citrate (lane 6). Products were resolved by TLC. Chloramphenicol (CM) and its acetylated forms (A) 3-acetate chloramphenicol, (B) 1-acetate chloramphenicol, and (C) chloramphenicol were detected by autoradiography. The 1,3-diacetate chloramphenicol (not shown in this autoradiogram as its radioactive content is low) moves most rapidly above row A. Lane 1, no added CL; lanes 2−5, increasing amounts of CL. Samples in lanes 1−5 (panel i) incubated with [^{14}C]citrate. Samples in lanes 6−10 (panel ii) incubated with increasing amounts of CL with [^{14}C]chloramphenicol. Lane 11, acetyl-CoA transferred to [^{14}C]chloramphenicol with CAT. CL that assayed at 0.5 unit (pH 7.5) was added to incubations as indicated. Lanes 1−5 and 6−10: 0, 19.4, 38.8, 58.1, and 77.5 μg, respectively. Citrate cleaved or acetyl-CoA formed is, respectively, 0, 0.53, 0.87, 1.04, and 1.14 and 0, 0.71, 0.95, 1.12, and 1.30 nmol/min for samples in lanes 1−10. Values are calculated from radioactive contents of spots visualized by autoradiography. The radioactivity associated with spot (lane 6) is approximately equal to the total radioactivity recovered in spots in lanes 7−10.

μL which is added to scintillation fluid and assayed for radioactivity. As one carbon from citrate is used to synthesize acetyl-CoA and the other carbon to synthesize OA, the number of added cpm to an assay is halved in the final calculations. One unit of activity is defined as 1 μmol of citrate cleaved or acetyl-CoA formed (mg of protein)$^{-1}$ min^{-1} at 37 °C. Results of this assay were similar whether CAT and chloramphenicol were added at the beginning or at the conclusion of an assay (after brief heating, followed by continued incubation at 25 °C for 10 min).

To validate this procedure, products of the CL−citrate coupled CAT assay were resolved by TLC. The residue (lyophilized ethyl acetate extract) was dissolved in 50−100 μL of chloroform. The sample was applied to a precoated silica gel 60 TLC glass plate, and products were resolved by ascending chromatography using chloroform/methanol (90/10) as solvent. The results obtained were identical to those of Gorman et al. (1982), producing, in order of increasing mobility, 1-acetate chloramphenicol, 3-acetate chloramphenicol, and 1,3-diacetate chloramphenicol. The 3-acetate form is most abundant whereas the 1,3-diacetate form being least abundant is difficult to detect.

Cells and Cell Culture Conditions. The schema for 3T3-L1 cell differentiation (Rubin et al., 1978) is fully described in our recent publication (Benjamin et al., 1994). Adipocytes, 6−8 days after reaching confluency, are swollen and filled with fat droplets.

CL Phosphorylation in Vitro. Rat liver CL (100 μg) was phosphorylated for 4 h at 37 °C, with cAMP-dependent protein kinase or this kinase plus GSK-3 in buffer (total volume 100 μL) consisting of 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, and 2.5 mM ATP. The control and phosphorylated samples are preincubated with 10 mM DTT for 20 min (Wells, 1991) and assayed immediately for CL activity by the CL−citrate coupled CAT assay. An aliquot is frozen, and later subunits of the CL holoenzyme (150 ng) are resolved by SDS−PAGE[1] (7% acrylamide), gel dimensions 13.5 × 14.7 × 0.12 cm, at a constant voltage of 90 V for 16 h. Electrotransfer of proteins to nitrocellulose and complete description of Western blotting using ECL are as described (Benjamin et al., 1994).

Kinetic Studies. ATP-citrate lyase activity was assayed using the new CAT assay. The amounts of Mg•ATP and Mg•citrate for each experimental condition were calculated using their dissociation constants as described by Plowman and Cleland (1967). Initial rates for the enzyme reaction with respect to Mg•ATP, Mg•citrate, and CoA were obtained by applying a quadratic fit (Curve fitter method) directly to the initial velocities. The V_{max} and K_m values for each experimental point were calculated by the nonlinear curve fit method using the Michaelis−Menten equation. Double-reciprocal plots were generated by the weighted least mean-squares procedure. The mean K_m values and standard errors for the data were calculated by Sigma Plot (Version 2.0) statistical analysis function.

RESULTS

In the CL−citrate coupled CAT assay, chloramphenicol is acetylated by added CAT using enzymatically synthesized acetyl-CoA as substrate. To show this synthesis of acetyl-chloramphenicol, [^{14}C]citrate was incubated with CL. The lipid-soluble products of this reaction were lyophilized, dissolved in chloroform, applied to a TLC plate, and resolved as described (Figure 1). Radioactive spots (determined by autoradiography) were scraped, and radioactivity was measured. As controls, acetyl-CoA was incubated with [^{14}C]-chloramphenicol and CAT, or samples were incubated either with or without added citrate, enzyme, or ATP.

Figure 1 shows an autoradiogram of the chromatogram by TLC of the products of the CL reaction. Row A corresponds to 3-acetate chloramphenicol and row B to 1-acetate chloramphenicol. The 1,3-diacetate chloramphenicol spot containing little radioactivity is not shown in this figure. When citrate is not added (lane 6), the major radioactive product of a reaction mixture containing CL and [^{14}C]chloramphenicol runs near the origin similar to the [^{14}C]-chloramphenicol standard. When citrate is added to a reaction mixture containing [^{14}C]chloramphenicol but without added CAT, the radioactive product also runs with marker radiolabeled chloramphenicol (data not shown). Products of assays with added acetyl-CoA, [^{14}C]chloramphenicol, and CAT but without added CL (lane 11) chromatograph with the acetylated chloramphenicols. The effects of adding increasing amounts of CL on acetyl-CoA synthesis are shown in lanes 1−5. No radioactive product is extracted into the organic phase when CL is left out of an assay (lane 1). With increasing amounts of added CL, increasing amounts of radioactivity are resolved by TLC into discrete spots with the mobility of radiolabeled acetylchloramphenicols. In confirmation of these results, when limiting (0.3 mM) amounts of [^{14}C]chloramphenicol are added to a reaction mixture with unlabeled citrate and increasing amounts of CL, products of the reaction contain increasing amounts of radioactivity that run at the same positions as acetylated chloramphenicol species. There is a proportional decrease

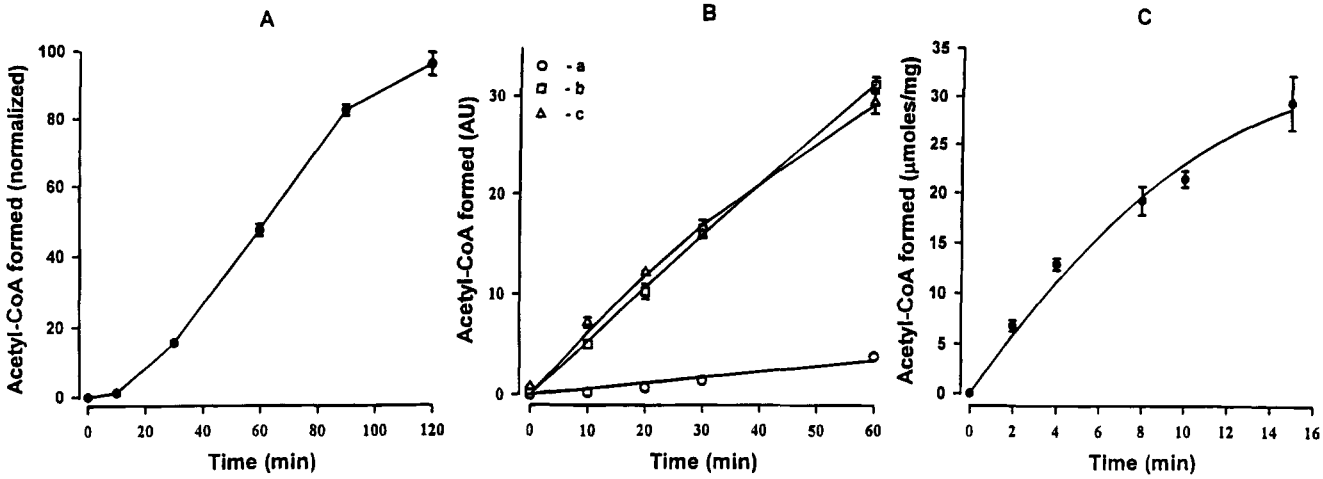

FIGURE 2: CL activity. (A) No DTT pretreatment. Activity was measured by the CL−citrate coupled CAT assay. Each assay containing Mg•citrate, 0.9 mM; Mg•ATP, 2.5 mM; $MgSO_4$, 10 mM; DTT, 10 mM; CoA, 0.33 mM; and HEPES, 50 mM (pH 7.5), was incubated at 37 °C for the time indicated. Each observation is the mean ± SE of a minimum of three independent measurements performed twice using two different CL preparations. For each assay, the micromoles of acetyl-CoA formed at 120 min was set at 100. The values obtained (within each experimental group) at earlier time points were normalized to the measurement at the maximum time. (B) DTT treatment. CL activated with 1 mM (a) or 10 mM (b and c) DTT for 20 min was assayed at a final DTT concentration as indicated in the figure: 1 mM (a); 1.6 mM (b); or 11.6 mM (c). Each assay containing Mg•citrate, 0.9 mM; Mg•ATP, 2.5 mM; $MgSO_4$, 10 mM; DTT, as indicated; CoA, 0.33 mM; and HEPES, 50 mM (pH 7.5), was incubated at 37 °C for 1 h. Each observation is the mean ± SE of three independent measurements performed twice using two different CL preparations and is expressed in arbitrary units. As these two CL preparations were of similar activity, and when assayed at pH 7.5 assay at a lower value than at pH 8.7, the data are expressed as 1 arbitrary unit (AU) = 0.1 μmol of acetyl-CoA formed/mg of protein. (C) CL activity at early time points. Using a different CL preparation than in panel A or panel B, enzyme was activated with 10 mM DTT for 20 min. CL was assayed in a reaction mixture containing (final concentration) Mg•citrate, 1.8 mM; Mg•ATP, 2.5 mM; $MgSO_4$, 10 mM; DTT, 10 mM; CoA, 0.66 mM; and Tris•HCl, 50 mM (pH 8.7), at 37 °C for varying times. Each observation is the mean ± SE of three independent measurements.

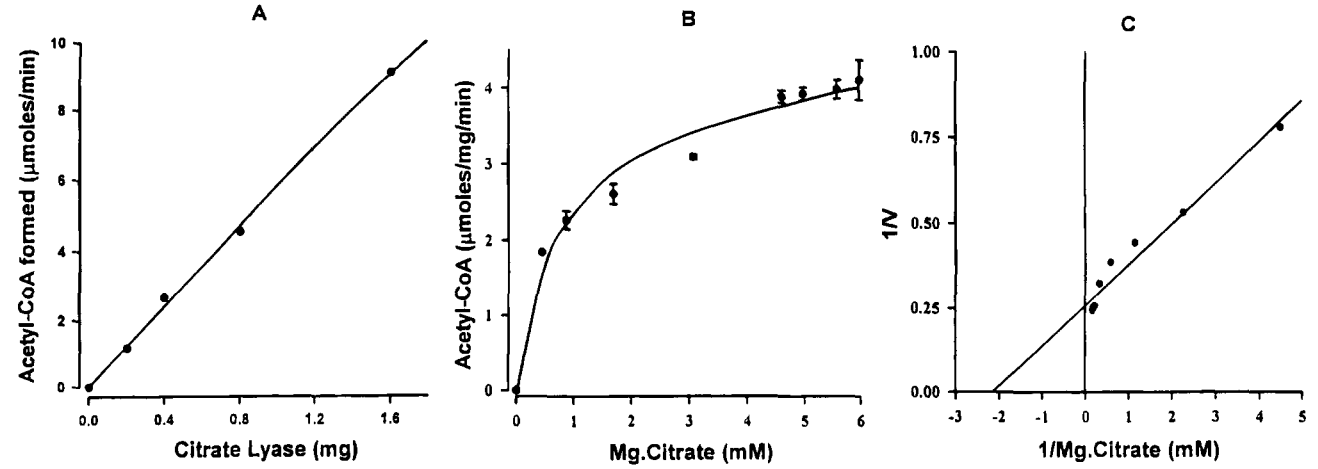

FIGURE 3: CL activity. (A) Acetyl-CoA synthesized as a function of added CL. Activity was measured by the CL−citrate coupled CAT assay. CL was activated with 10 mM DTT as described. Each assay containing Mg•citrate, 4.2 mM; Mg•ATP, 2.4 mM; $MgSO_4$, 10 mM; DTT, 10 mM; CoA, 0.66 mM; Tris•HCl, 50 mM (pH 8.7); and amounts of CL as indicated was incubated for 10 min at 37 °C. (B) Acetyl-CoA synthesized as a function of citrate concentration. CL was activated with 10 mM DTT. Each assay containing CL, 0.98 μg; Mg•citrate, 0−6 mM; Mg•ATP, 2.4−2.5 mM; $MgSO_4$, 10 mM; DTT, 10 mM; CoA, 0.66 mM; and Tris•HCl, 50 mM (pH 8.7), was incubated for 10 min at 37 °C. Each measurement is the mean ± SE of three independent observations using purified enzyme that assayed at 3.8 units. (C) Lineweaver−Burk plot. Double-reciprocal plot of values in panel B at varying Mg•citrate concentrations.

in radioactivity associated with substrate chloramphenicol with increasing amounts of added CL (lanes 6−11). As acetyl-CoA synthesis increases with added enzyme, acetylation consumes the limited amount of added [^{14}C]chloramphenicol. The total radioactivity content (lane 6) is approximately equal to the total radioactivity of rows A−C (lanes 7−10), indicating that the increased transfer of acetyl-CoA to chloramphenicol is a function of added CL. As it was demonstrated that products of the CAT reaction are quantitatively extracted into the organic phase, this assay requires only analysis of the organic phase for radioactive content to measure CL activity. In support of this conclusion, no radiolabeled chloramphenicol or its acetylated products were found in the aqueous phase after extraction, and when radiolabeled acetyl-CoA was added to an incubation (without added CL), it was almost completely converted to acetyl-chloramphenicol (data not shown) when CAT and chloramphenicol were added in excess, showing that the products were extractable by ethyl acetate.

In this TLC autoradiogram (Figure 1), citrate concentrations were decreased to maintain high specific activity for autoradiography, and therefore synthesis of acetyl-CoA was not a linear function of added CL. As shown in Figure 3A, when Mg•citrate concentrations are close to optimal and

Table 1: Enzyme Kinetics of ATP-Citrate Lyase: Kinetic Constants[a]

varied reactant	K_m (mM)
Mg·citrate	0.454 ± 0.04
Mg·ATP	0.042 ± 0.004
CoA	0.044 ± 0.002

[a] Activated CL enzyme activity was measured using the CL−citrate coupled CAT assay at various concentrations of Mg·citrate, Mg·ATP, and CoA. Activated CL [3.85 units; unit = micromoles of acetyl-CoA formed per milligram of protein per minute at 37 °C] was assayed for 10 min. Computational analyses (curve fitter method) of the relation between reactants and CL activity were drawn and apparent K_m values for reactants calculated. Each observation is the mean ± SE of a minimum of three independent observations.

lower amounts of CL enzyme are added, the CL−citrate coupled CAT assay measures the rate of citrate cleavage as a linear function of added enzyme.

CL is reversibly inactivated by oxidation and may take time to fully activate with treatment by 2-mercaptoethanol (Wells, 1991; Wells & Saxty, 1992) when measured by the MDH assay. This phenomenon is also found using the CL−citrate coupled CAT assay to measure CL activity. Without DTT pretreatment, CL activity is both decreased and not linear (Figure 2A,B). With preincubation of enzyme with 10 mM DTT, CL activity is markedly increased, and acetyl-CoA formation increases linearly with time. Incubation at 1 mM DTT for 30 min (data not shown) is not sufficient to correct these defects, and activity remains less than maximal even during long incubation times at the lower DTT concentrations (Figure 2B). Preincubation at 10 mM DTT activates the enzyme, and maximal activity persists during an assay, even when DTT concentrations are lowered to 1.6 mM. The CL−citrate coupled CAT assay gave similar specific activity values to those obtained using the MDH assay for each purified CL preparation studied.

Four different rat liver CL preparations were purified through DE-52 chromatography. Three preparations were further purified by Affigel chromatography (Wells, 1991). Using either the MDH or the CL−citrate coupled CAT assays, these preparations assayed between 0.5 and 4 units at pH 7.5 and up to 8.0 units at pH 8.7. A representative plot of acetyl-CoA formed as a function of added CL is shown in Figure 3A. At a more optimal Mg·citrate concentration and with lower amounts of added CL protein (as in these studies), the rate of acetyl-CoA formation is linear with respect to added enzyme.

Acetyl-CoA formed as a function of Mg·citrate concentration and the Lineweaver−Burk analyses of these data are shown in Figure 3B,C. The rate of acetyl-CoA production by CL rapidly increases with increasing Mg·citrate concentration (Figure 3B) and plateaus above 4 mM Mg·citrate. The apparent K_m for Mg·citrate is 0.42 mM (Figure 3C and Table 1).

CL activity, as a function of Mg·citrate, Mg·ATP, and CoA concentrations, using different enzyme preparations and assayed at either pH 7.5 or pH 8.7, is shown in Table 1. CL activity increases with increasing Mg·citrate, Mg·ATP, and CoA concentrations. At 4 mM Mg·citrate, the rate of acetyl-CoA synthesis plateaus at ATP concentrations above 0.5 mM and CoA concentrations above 0.165 mM. These kinetic parameters, which are similar to those determined for CL using the MDH assay (Takeda et al., 1969; Singh et al., 1976; Ramakrishna & Benjamin, 1979; Houston & Nimmo, 1985a,b), are shown in Table 1.

The effects of other possible regulators on CL activity were also studied.[2] OA, a product of the reaction, was found to significantly inhibit purified CL activity (Figure 4A). This finding was confirmed using different enzyme preparations, assayed at either pH 7.5 or pH 8.7, at varying OA concentrations. Lineweaver−Burk analyses (Figure 4B) show that the apparent V_{max} decreases with increasing OA concentration whereas the apparent K_m for Mg·citrate does not change significantly. The percent decrease in the apparent V_{max} and the absence of change in the apparent K_m for citrate are summarized in Table 2. Inhibition is over a range of OA concentrations (0.25−4.0 mM).

In 3T3-L1 fat cells, most CL is phosphorylated in complex forms that are under hormonal control (Benjamin et al., 1994). To determine whether phosphorylation affects enzyme activity, purified CL was phosphorylated *in vitro* and its activity determined. CL was phosphorylated by cAMP-dependent protein kinase or by this protein kinase and GSK-3. Control and phosphorylated enzymes were resolved into subunits by SDS−PAGE and visualized by Western blotting (Figure 5A, insert). As recently demonstrated (Benjamin et al., 1994) and as shown for this study, CL prepared for these assays migrates on gel electrophoresis as a function of its phosphorylation state. By using a low amount of CL protein in the phosphorylation reaction (100 μg), CL is phosphorylated so that almost all protein subunits migrate more slowly and are in a phosphorylated form.

Enzyme activities of two different CL preparations were studied over a range of Mg·citrate concentrations. Lineweaver−Burk analyses of these data are shown in Figure 5B. The data from experiments using both preparations were calculated as described in the legends to Figure 5 and Table 2. *In vitro* phosphorylation does not change the apparent K_m for Mg·citrate but does reduce the apparent V_{max} (Figure 5 and Table 2).

To determine whether OA inhibited CL activity as a function of its phosphorylation state, three CL preparations that were first phosphorylated *in vitro* were studied over a range of Mg·citrate concentrations at 0 and 2 mM OA. CL phosphorylation decreased the apparent V_{max} but did not change the apparent K_m for citrate when compared to control (Table 2). OA was again found to be an inhibitor with respect to citrate for all forms of the enzyme tested, decreasing the apparent V_{max} without affecting the apparent K_m. However, OA did not distinguish between control and phosphorylated forms as the percent decrease in activity by OA, compared to its unphosphorylated or phosphorylated control, was similar in all samples tested. Additional assays were performed to determine the effect of phosphorylation on OA inhibition of CL activity by measuring its apparent K_i (Dixon, 1953). The apparent K_i for OA (Table 3) did not change with CL phosphorylation, and therefore OA and CL phosphorylation had an additive and not a synergistic effect in decreasing the apparent V_{max}.

As the CL−citrate coupled CAT assay is able to measure CL activity in biological samples, the effect of OA on activity was measured in cytosol extracts from 3T3-L1 fat cells. OA

[2] Adenosine 5-monophosphate (<1 mM), malonyl-CoA (10−500 μM), and palmityl-CoA (0.5−100 μM) had no effect on CL activity (purified enzyme).

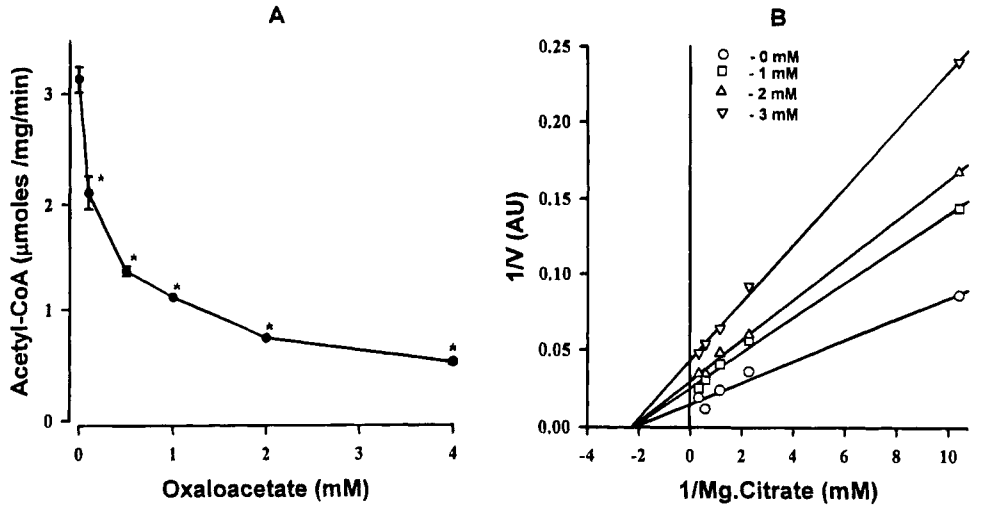

FIGURE 4: CL activity. (A) Effect of OA. CL activity was measured at various concentrations of OA. Each assay containing CL, 0.84 μg; Mg•citrate, 3.5 mM; Mg•ATP, 2.4 mM; $MgSO_4$, 10 mM; DTT, 10 mM; CoA, 0.66 mM; and Tris•HCl, 50 mM (pH 8.7), was incubated for 10 min at 37 °C. Values are the mean ± SE of three independent observations. Asterisk, significantly different from control at $p < 0.05$. (B) Lineweaver–Burk plot. CL activity was assayed at various citrate concentrations at 0, 1, 2, and 3 mM OA. Each assay containing Mg•ATP, 2.4–2.5 mM; CoA, 0.33 mM; $MgSO_4$, 10 mM; DTT, 10 mM; and HEPES, 50 mM (pH 7.5), was incubated for 1 h at 37 °C. These results are from an independent set of experiments using a different preparation of CL (assayed at pH 7.5) from those in panel A and are expressed in arbitrary units. 1 AU = 0.1 μmol (mg of protein)$^{-1}$ h^{-1}.

Table 2: Effect of Phosphorylation and Oxaloacetate on CL Enzyme Activity[a]

	% decrease in appV_{max}	appK_m for Mg•citrate
control (6.25 units)[b]	24.9	0.45 ± 0.04
1 mM OA	24.9	0.50 ± 0.06
2 mM OA	43.6	0.44 ± 0.03
3 mM OA	62.3	0.48 ± 0.08
control	− (46.6)	0.45 ± 0.08
A-kinase	29.2 (53.1)	0.45 ± 0.08
A-kinase + GSK-3	40.9 (48.7)	0.48 ± 0.04

[a] Phosphorylated (A-kinase and A-kinase- and GSK-3-treated samples) and control CL preparations (activated before enzyme assay with 10 mM DTT for 20 min) were assayed with various concentrations of Mg•citrate with and without OA, 2 mM. Each assay containing DTT, 10 mM; CoA, 0.33 mM; Mg•ATP, 2.4–2.5 mM; magnesium sulfate, 10 mM; and Tris•HCl, 50 mM (pH 8.7), was incubated at 37 °C. Apparent K_m values for Mg•citrate (mM) were calculated. Values are the percent decrease in the apparent V_{max} due to phosphorylation, and the numbers in parentheses are the additional percent decrease in the apparent V_{max} of the control (incubated for 4 h without added kinase) and phosphorylated samples incubated with 2 mM OA. [b] Micromoles of acetyl-CoA formed per milligram of protein per minute at 37 °C.

was found to be an inhibitor of CL activity (Figure 6A). Its apparent K_i is significantly lower, 63 μM, than that found for the pure enzyme, 1.2 mM (Table 3). CL activity was assayed at different Mg•citrate concentrations at 0 and 0.1 mM OA. Lineweaver–Burk analyses of these data show that the apparent K_m of citrate for CL in cytosol is similar to purified CL. Again, the apparent V_{max} is decreased by OA (Figure 6B).

DISCUSSION

The mechanism of citrate cleavage by CL, a large enzyme composed of identical subunits, is complex, multistep, and not fully understood (Wells, 1991). A beneficial reason for multisubunit enzymes, particularly if identical, is thought to be that subunits cooperate in catalysis (Kayalar et al., 1973). Accordingly, catalysis at one active site may be promoted by transformations that result from conformational changes on the same or adjacent subunits producing transient asymmetries in the configuration of otherwise identical subunits. An enzyme, structurally similar to ATP-citrate lyase, succinyl-CoA synthetase (Elshourbagy et al., 1990), appears to operate via alternating sites that are catalytically cooperative (Wolodko, 1983). CL, though possibly possessing the requisites to operate similarly, has not been shown to rapidly regulate its activity via any mechanism including phosphorylation or allosterism. CL has not been thought to be important in short-term regulation of lipid metabolism. This paper reports studies using a new CL assay which reexplore the question whether there is rapid regulation of CL by small molecules or covalent modifications.

Studies suggest (Plowman & Cleland, 1967; Houston & Nimmo, 1984) that CL follows a double-displacement mechanism. Wells (1991) shows that there is no requirement for the mechanism to proceed via a covalent citryl-enzyme intermediate. As has been shown for succinyl-CoA synthetase, when CL is incubated with the citrate and CoA, rather than forming a covalent citryl-enzyme intermediate complex, citryl phosphate forms without such an antecedent complex (Wells, 1991). Evaluation of some of the CL kinetic curves presented in this paper suggests that its reaction mechanism is complex, and further studies on the mechanism are left for future investigation.

The MDH assay is not satisfactory to measure CL activity in cell extracts as uncontrolled substances oxidize NADH in the absence of ATP, citrate, or added MDH. The previously described hydroxymate chemical-color method or the cleavage of [^{14}C]citrate to OA with conversion of OA to $^{14}CO_2$ (Takeda et al., 1969) were either too insensitive or cumbersome to be widely used to study CL activity in biological samples. A new CL activity assay was developed using the CAT procedure (Gorman et al., 1982) to couple acetyl-CoA production to chloramphenicol acetylation, an easily measurable event. This assay measures acetyl-CoA production from citrate by CL. Acetyl-CoA production is linear for over 2 h of incubation and depends on the amount of CL added (Figures 1–3). Production of acetyl-CoA depends on the addition of Mg•citrate, Mg•ATP, and CoA,

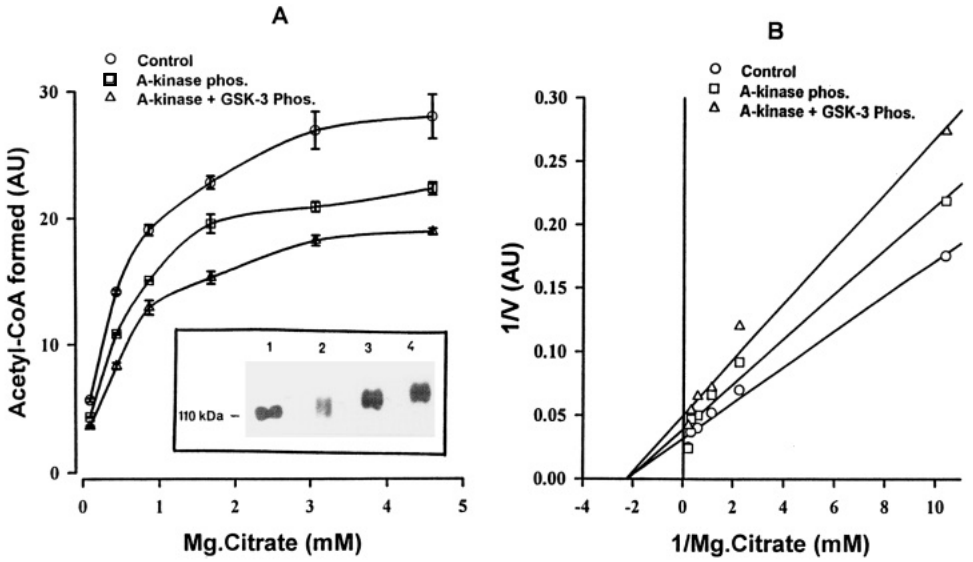

FIGURE 5: CL activity. (A) Effect of CL phosphorylation. Activated CL as control or phosphorylated by cAMP-dependent protein kinase or this kinase plus GSK-3 was assayed at various citrate concentrations. Each assay containing CL, 1.3 or 1.8 μg; Mg·ATP, 2.4−2.5 mM; CoA, 0.33 mM; $MgSO_4$, 10 mM; DTT, 10 mM; and HEPES, 50 mM (pH 7.5), was incubated for 1 h at 37 °C. Values are the mean ± SE of three independent observations using two different enzyme preparations expressed in arbitrary units. 1 AU = 0.1 μmol (mg of protein)$^{-1}$ h^{-1}. Insert: Western blots of purified CL phosphorylated *in vitro* by A-kinase and GSK-3. Rat liver CL (100 μg) purified without addition of phosphatase inhibitors was phosphorylated by cAMP-dependent protein kinase or this kinase and GSK-3 in buffer, total volume of 100 μL, containing HEPES, 50 mM (pH 7.5); $MgCl_2$, 10 mM; DTT, 1 mM; and Mg·ATP, 2.5 mM, at 37 °C for 4 h. Subunits of CL protein (150 ng) were resolved by SDS−PAGE. Lanes 1−4, immunoblots. Lane 1, Cytosol CL (20 μg of protein) from 3T3-L1 confluent cells which contain mostly the dephospho form of the enzyme (Benjamin et al., 1994) was electrophoresed close to experimental samples as an *in vivo* CL marker for the 110 kDa CL position; 2, CL incubated for 4 h without added kinases; 3, cAMP-dependent protein kinase phosphorylated CL; 4, cAMP-dependent protein kinase and GSK-3 phosphorylated CL. (B) Lineweaver−Burk plot. Double-reciprocal plot of values in panel A at varying Mg·citrate concentrations.

Table 3: Effect of OA on CL Activity (K_i)[a]

enzyme	K_i
CL (control)	1.17 mM
CL (incubated without kinases)[b]	1.21 mM
CL (A-kinase + GSK-3)[b]	1.17 mM
CL (GSK-3)[b]	1.23 mM
CL (cytosol of 3T3-L1 adipocytes)	63.0 μM

[a] Phosphorylated and control CL and cytosol from 3T3-L1 adipocytes were assayed with various concentrations of OA. The reaction mixture contained Tris·HCl, 50 mM (pH 8.7); Mg·citrate, 3.5 mM; Mg·citrate, 3.5 mM; Mg·ATP, 2.4 mM; $MgSO_4$, 10 mM; DTT, 10 mM; and CoA, 0.66 mM, at 37 °C for 10 min. K_i values were calculated according to the method of Dixon (1953). [b] Incubated for 4 h as described under Experimental Procedures before activation with DTT and assay.

and kinetic constants for these reactants (Table 1) are similar to those determined in prior studies using the MDH assay (Takeda et al., 1969; Singh et al., 1976; Ramakrishna & Benjamin, 1979; Houston & Nimmo, 1985a). The effects of pH and reducing agent treatments on CL activity are similar to those reported using the MDH assay (Figure 2). The CL−citrate coupled CAT assay is at least 10 times more sensitive than the MDH assay and is able to measure CL activity in unprocessed biological samples.

The effect of OA on CL activity using purified enzyme was determined (Figure 4). This previously had not been possible because the MDH assay consumes the OA produced to synthesize malate and oxidize NADH. Surprisingly, using purified enzyme, OA was found to be an inhibitor of the enzyme. Inhibitions by OA were at concentrations (Figure 4 and Tables 2 and 3) that are higher than those reported for OA in liver cells as cellular OA concentrations are estimated to be between 10 and 20 μM (Rawat, 1968). The OA inhibition had an apparent K_i of approximately 1.2 mM though significant effects are found at 0.25 mM. OA decreased the apparent V_{max} of the enzyme without changing the apparent K_m for Mg·citrate (Figure 4B). OA appears not to displace citrate from its catalytic site, and its mechanism of inhibition is unknown.

The CL−citrate coupled CAT assay is also able to measure CL activity in the cytosol of 3T3-L1 fat cells (Figure 6A). The apparent K_m for Mg·citrate in cytosol is similar to that of purified enzyme. CL activity measured in cytosol is more sensitive to OA inhibition than is purified enzyme, with effects seen at 25−50 μM (Figure 6A and Table 3). The reason(s) why cytosolic CL is more sensitive to OA inhibition is (are) unknown but may include the following: (1) *in vivo* phosphorylation of the holoenzyme may not be duplicated by *in vitro* phosphorylation; (2) an allosteric effector that sensitizes the enzyme to OA inhibition may be lost during purification; (3) subtle degradation of the enzyme (proteolysis) may occur during purification. The increased sensitivity of cytosolic CL to OA is most likely not due to the OA contained in the added cytosol as the final dilution of the cytosol in an assay is at least 60 times, rendering the added OA insignificant.

CL, a major enzyme in fat biosynthesis (Sullivan et al., 1973), is important in long-term (hours to days) control of lipid synthesis in fat and liver tissues. However, it is believed that rapid regulation of fat synthesis resides principally at the next enzyme in fat synthesis, ACC (Munday et al., 1988; Haystead & Hardie, 1988; Haystead et al., 1988; Witters et al., 1988), where AMP and citrate concentrations, and ACC phosphorylations, are important in short-term regulation of its activity (Davies et al., 1990). Certain facts about CL, however, suggest that its activity could also be controlled. Among these are the following: (1) the complex phosphorylation state of CL in fat and liver tissues is rapidly and

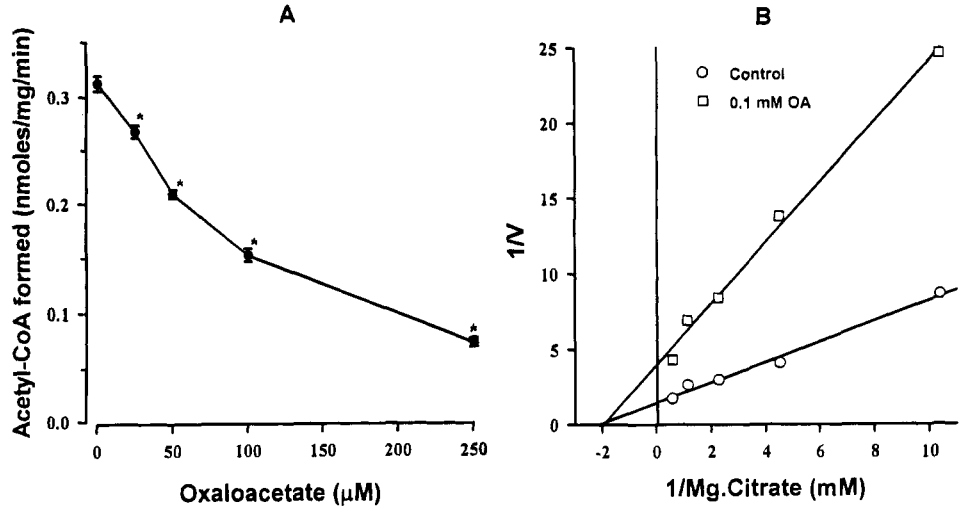

FIGURE 6: Effect of OA on CL activity in 3T3-L1 fat cell cytosol. (A) Inhibition by OA. Cytosol from fat cells was assayed for CL enzyme activity after activation for 20 min with 10 mM DTT at various concentrations of OA. Incubations were for 1 h using 20 μg of cytosolic protein. Each assay containing Mg·citrate, 2.2 mM; Mg·ATP, 2.5 mM; $MgSO_4$, 10 mM; DTT, 10 mM; CoA, 0.33 mM; and HEPES, 50 mM (pH 7.5), was incubated at 37 °C. Each observation is the mean ± SE of three independent observations. Asterisk, significantly different from control at $p < 0.05$. (B) Lineweaver–Burk plot. At various concentrations of substrate (citrate), cytosol from fat cells was assayed at 0 and 0.1 mM OA. OA at 0.1 mM had been determined to decrease enzyme activity by approximately 50%. Incubations were for 1 h using 20 μg of cytosolic protein after activation of cytosol with 10 mM DTT for 20 min. Each assay containing DTT, 10 mM; CoA, 0.33 mM; Mg·ATP, 2.4–2.5 mM; magnesium sulfate, 10 mM; and HEPES, 50 mM (pH 7.5), was incubated at 37 °C. Each value is the mean ± SE of three independent observations.

substantially altered in unique patterns by changes in hormone concentrations and metabolic states (Ramakrishna et al., 1989, 1990; Benjamin et al., 1994); (2) these phosphorylation patterns and their control by hormones closely resemble glycogen synthase's phosphorylation patterns at site 3 that control enzyme activity (Ramakrishna et al., 1990); (3) the protein kinase (GSK-3) that phosphorylates glycogen synthase at site 3, decreasing its enzyme activity in the absence of glucose 6-phosphate, is the same kinase that phosphorylates CL at residues 446 and 450 (Ramakrishna et al., 1990; Hughes et al., 1992); (4) in fat tissue (Ramakrishna & Benjamin, 1988), CHO cells (Welsh & Proud, 1993), and 3T3-L1 adipocytes (Benjamin et al., 1994), GSK-3 activity and/or content fall with insulin stimulation or fat cell differentiation. Furthermore, it has recently been shown that GSK-3 activity falls in A431 cells stimulated by epidermal growth factor (Saito et al., 1994), adding to the belief that this is a general mechanism.

Kinetic studies (this paper) on *in vitro* phosphorylated CL demonstrate that phosphorylated CL, compared to control, shows decreases in the apparent V_{max} without significant changes in the apparent K_m (Figure 5, Table 2). Reasons why these reproducible changes in CL activity, as a function of enzyme phosphorylation, were not found previously are not known. Possible explanations for this failure are as follows: (1) In the CL–citrate coupled CAT assay, OA synthesized by the enzyme is not consumed by added MDH. This may permit CL phosphorylations to affect activity whereas, in the MDH assay, OA concentration always approaches zero. (2) The addition of MDH and NADH, with their associated salts (NH_4SO_4), may affect the enzyme so that phosphorylation effects on activity are nullified. (3) The CL–citrate coupled CAT assay is more sensitive than the MDH assay. Because the new assay uses much less CL (protein), enzyme subunit association/disassociation may be affected. These possibilities have not been explored but provide a framework for further study of the complicated enzymology of CL.

To determine whether phosphorylations change CL sensitivity to OA inhibition, the effect of OA on activity in control and two phosphorylated CL preparations was studied (Tables 2 and 3). OA inhibited CL activity in all samples studied. No differential sensitivity to OA between CL phospho-states and control was found as OA inhibited these samples equally. Decreases in CL activity produced by OA and phosphorylation were additive.

In this paper, we report that the apparent V_{max} decreases in phosphorylated CL compared to control without change in the apparent K_m. In addition, OA was found to be an inhibitor of both purified CL and CL activity in cytosol. These kinetic changes (in cytosol) occur at Mg·citrate and OA concentrations that are low and for citrate within the physiological range (Rawat, 1968).

Regulation of CL activity by phosphorylation and OA appears to be graded rather than all-or-none. This graded control system may be advantageous because of a possible dual function for CL in fat and liver cells. In these tissues, CL provides a carbon source for fat and cholesterol synthesis during anabolism but could also provide a carbon source for gluconeogenesis and substrate for fatty acid esterification during catabolism. Study of changes in citrate and OA concentrations as a function of insulin or β-adrenergic agent treatment or cell starvation with measures of CL activity and content are underway to determine if these new findings that show changes in CL activity as a function of CL phosphorylations and allosteric effectors are physiologically relevant.

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