Inhibition of Acetyl-CoA Carboxylase Isoforms by Pyridoxal Phosphate*

Weissy M. Lee, Jason E. Elliott, and Roger W. Brownsey

Mammalian isoforms of acetyl-CoA carboxylase (ACC-1 and ACC-2) play important roles in synthesis, elongation, and oxidation of long-chain fatty acids, and the possible significance of ACC in the development of obesity has led to interest in the development of inhibitors. Here, we demonstrate that pyridoxal phosphate (PLP) is a linear and reversible inhibitor of ACC-1 and ACC-2. ACC from rat liver and white adipose tissue (largely ACC-1) exhibited an IC50 of ~200 μM, whereas ACC-2 from heart or skeletal muscle exhibited an IC50 exceeding 500 μM. ACC from rat liver was equally sensitive to PLP following extensive purification by avidin affinity chromatography. When added before citrate, PLP inhibited ACC with a Ki of ~100 μM, reducing maximal activity >90% and increasing the Km for citrate ~5-fold but having little effect on substrate Km values. Pre-treatment with citrate increased the apparent Ki for ACC inhibition by PLP by ~4-fold. Inhibition of ACC was reversed by removal of PLP, either by washing or by reaction with hydroxylamine or amino-oxacetate. ACC was irreversibly inhibited and radiolabeled, to a stoichiometry of ~0.4 mol [H]/mol subunit, in the presence of PLP plus [3H]borohydride. Studies with structurally related compounds demonstrated that the reactive aldehyde and negatively charged substrates of PLP contribute importantly to ACC inhibition. The studies reported here suggest a rationale to develop ACC inhibitors that are not structurally related to the substrates or products of the reaction and an approach to probe the citrate-binding site of the enzyme.

Acetyl-CoA carboxylase (ACC, EC 6.4.1.2) catalyzes the biotin-dependent formation of malonyl-CoA and was discovered as a result of studies that defined the requirements for fatty acid synthesis in cell-free extracts (1, 2). Many of the fundamental properties of ACC were initially established with studies of chicken and rat liver forms of ACC (3, 4). Subsequently, it has been recognized that two major mammalian ACC isoforms exist. The gene for ACC-1 (ACC-α) encodes a protein with subunit Mr 265,000 that is expressed at high levels in lipogenic tissues, notably in fat, liver, and lactating mammary gland (5–8). ACC-2 (ACC-β) is closely related to ACC-1 but exhibits a larger subunit size of Mr 280,000 and is encoded by a separate gene (9, 10). Variants of both ACC isoforms are produced as a result of alternative splicing of mRNA (7–11). ACC-2 is a minor component of hepatic ACC but the predominant isoform, albeit expressed at relatively low levels, in heart and skeletal muscle (12–15). The presence of ACC-2 in non-lipogenic tissues provides support for the view that malonyl-CoA is essential not only as a substrate for fatty acid synthase, but also for controlling fatty acid oxidation, by inhibiting carnitine palmitoyl transferase-I (16–19). Indeed, ACC is also required for fatty acyl chain elongation (20) and may have additional roles resulting from association with other proteins such as BRCA1 (21).

The expression of ACC isoforms is tissue-specific and is controlled during development and in response to hormones or changes in dietary composition (3, 22–24). ACC is also sensitive to allosteric ligands and subject to multiple-site phosphorylation, mechanisms that facilitate rapid responses to metabolic demands and cellular stresses (25–28). The observation that fatty acid synthesis is stimulated by intermediates of the tricarboxylic acid cycle (29) led to the discovery that ACC is directly activated by citrate (30, 31). In fact, all animal forms of ACC so far tested are activated by citrate, including those from adipose tissue (30), liver (3, 31), mammary gland (32), heart (12), and skeletal muscle (33). ACC activation is induced by physiologically relevant citrate concentrations, in the range 0.5–1 mM, provided the enzyme is not substantially phosphorylated (25–27). In skeletal muscle, cytosolic citrate concentration may change sufficiently to alter ACC activity (19, 34). In addition, the sensitivity of ACC to citrate is altered by phosphorylation. During ischemic stress, for example, ACC is rapidly phosphorylated by AMP-activated protein kinase and becomes refractory to the effects of citrate (35). The activation of ACC in response to insulin, or inhibition in response to glucagon or adrenaline, also correlates with changes in enzyme phosphorylation when tissue citrate concentrations appear to change very little (25–27).

In the absence of allosteric activators, ACC isoforms exist as dimers that can be discriminated into the component subunits only under harsh denaturing conditions. In concert with activation, citrate induces the association of ACC-1 dimers into large polymers detected as 45–55 S complexes by sucrose gradient centrifugation (36, 37) and visible as extended filaments by electron microscopy (38). ACC polymers have also been detected by sedimentation and size-exclusion analysis following insulin treatment of adipose tissue (39, 40). Despite its importance in the control of ACC activity, the site of interaction of citrate with the protein has not been defined. In searching for strategies to further define the effects of citrate on ACC, we noticed that pyridoxal phosphate (PLP) had been used to probe the citrate binding sites of several proteins, including phosphofructo-1-kinase (41–43) and mitochondrial carboxylate carriers (44, 45). In these cases, PLP was found to interact principally with specific lysyl residues within the citrate-binding site of the respective proteins. The studies described here were therefore designed to test the hypothesis that PLP might bind to and influence the activity of acetyl-CoA carboxylase.
EXPERIMENTAL PROCEDURES

Materials—Analytical grade solvents and chemicals were obtained from Fisher Scientific or BDH Chemicals (Vancouver, BC). HEPES, glycine, EDTA, EGTA, phenylmethylsulfonyl fluoride, ATP, PLP (and related compounds), glutathione, Pepstatin A, hydroxylamine, and amino-oxyacetate were from Sigma-Aldrich. Streptavidin-HRP, potassium [14C]bicarbonate, pre-stained high molecular weight Rainbow Markers, and ACS scintillation fluid were from GE Healthcare, formerly Amer- sham Biosciences. Other specific reagents were obtained as follows: fatty acid-free bovine serum albumin from ICN (Irvine, CA); coenzyme A from Roche Applied Science; Tris and MOPS from USB (Cleveland, OH); leupeptin from Peptides International (Louisville, KY); Ultrafree centrifugal filters from Millipore (Nepean, Ontario); SDS-PAGE reagents, gradient gels, and Affi-Gel-10 from Bio-Rad; hen egg-white avidin from Canadian Inovatech (Abbotsford, British Columbia); sodium [3H]borohydride from PerkinElmer Life Sciences (Woodbridge, Ontario); and SoftLink™ Soft Release Avidin Resin from Promega (Madison, WI).

Purification of ACC from Rat Tissues—Male Wistar rats weighing 150–250 g were obtained from the University of British Columbia animal care facility and handled according to the guidelines of the Canadian Council of Animal Care as approved by the UBC Committee on Animal Care. Rats were maintained on a 12-h light/dark cycle with free access to water and laboratory rat chow and killed by CO2 asphyxiation between 9:00 and 10:00 am, and tissues were removed for homogenization. Upon removal, liver was chilled on ice, skeletal muscle and heart were frozen in liquid nitrogen, and epididymal and peri-renal adipose tissues were dissected into small pieces (50–100 mg) and incubated for 30 min at 37 °C in Krebs-Henseleit buffer that had been pre-gassed with O2:CO2 (95%/5%, v/v). Following incubation, adipose tissue was blotted and frozen in liquid nitrogen.

Tissues were homogenized on ice in 20 mM MOPS buffer (pH 7.2), containing 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 2.5 mM benzamidine HCl, 3 μM Pepstatin A, 5 μM leupeptin, 2.5 mM glutathione, and 0.5 mM phenylmethylsulfonyl fluoride. Liver was diced into small pieces in 6 volumes of buffer and disrupted with a Potter-Elvehjem homogenizer. Frozen tissues were powdered under liquid nitrogen and then disrupted with a Polytron homogenizer (~5 s at setting #6) in the presence of 4, 9, or 5 volumes of homogenization buffer (muscle, heart, and adipose tissue, respectively). The homogenates were centrifuged at 4 °C for 90 s at 1000 × g, then for 30 min at 11,000 × g, and finally for 60 min at 215,000 × g, each supernatant being filtered through glass wool. Final high speed supernatants were adjusted to 40% saturation by addition of powdered ammonium sulfate, and the mixtures were stirred on ice for 1 h. Protein precipitates were collected by centrifugation (15,000 × g for 20 min) and then resuspended in homogenization buffer for storage at

![Graph A](image1.png)

**FIGURE 1.** Dose-dependent inhibition of ACC by PLP. ACC preparations were assayed after incubation (30 min) with the indicated concentrations of PLP, prior to (A and B) or following (C) the addition of sodium citrate (10 mM, 30 min). ACC was purified through ammonium sulfate precipitation from rat liver (●), heart (●), white adipose tissue (▲), and skeletal muscle (□). Liver ACC was also further purified by avidin-affinity chromatography (●, in C). The effects of adding PLP before (●) or after citrate (▲) were tested using rat liver ACC (●). Results are expressed as the mean ± S.E. for the 3–6 preparations or, for values without error bars, the average of two preparations. Activities are expressed relative to the maximum following incubation with 10 mM citrate in the absence of PLP. The specific activity of the enzyme preparations was 15–20 milliunits/mg of protein (liver), 30–40 milliunits/mg of protein (adipose tissue), and 0.5–2.5 milliunits/mg of protein (heart and skeletal muscle); that of affinity-purified ACC was 1.5 unit/mg of protein.
Inhibition of Acetyl-CoA Carboxylase

![Diagram](Image)

**FIGURE 2. Time-dependent inhibition of ACC by PLP.** Liver ACC was prepared by ammonium sulfate precipitation and PLP added during preincubation prior to assay (A) or only during enzyme assay (B). The addition of PLP (0.5 mM) during preincubation before assay was for the indicated times before (●) or after (○) incubation for 30 min with 20 mM citrate. When ACC was first activated with citrate (20 mM for 30 min, panel B), assays were carried out for the indicated times in the absence (●), or in the presence of 1 mM (●) or 5 mM PLP (○). Results are from one experiment that was repeated with very similar results.

first incubated with PLP and then further incubated with 10 mM citrate prior to assay (Fig. 1A). ACC from different rat tissues was studied to examine preparations with substantially differing ratios of the two major ACC isoforms. Previous studies have established that rat white adipose tissue contains exclusively ACC-1, heart and skeletal muscle contain predominantly ACC-2 (typically >90% of the total), and liver contains an ACC-1:ACC-2 ratio of ~3:1 to 4:1 (12–15, 33, 35). The same isoform patterns were confirmed in these studies by Western blotting using HRP-streptavidin (data not shown). ACC activity in each tissue preparation was inhibited by PLP in a concentration-dependent manner to a maximum extent of at least 90%. The IC_{50} for inhibition of ACC in extracts of liver and adipose tissue, largely or exclusively ACC-1, was 210 ± 30 μM and 200 ± 50 μM, respectively (mean ± S.E. for six enzyme preparations from each tissue). Assays of ACC from heart and skeletal muscle, predominantly ACC-2, gave IC_{50} values of 500 μM (mean of two preparations) and 620 ± 60 μM (mean ± S.E. for three preparations), respectively. Overall, IC_{50} values were significantly lower for preparations containing predominantly ACC-1 (205 ± 26 μM, n = 12), than for preparations in which ACC-2 is the dominant isoform (572 ± 46 μM, n = 5, p < 0.01). The calculated IC_{50} values are based on the concentration of PLP added during preincubation, rather than on the concentration in the final ACC assay mixture, which is 10-fold lower. ACC that had been further purified from rat liver by avidin affini-

RESULTS

**Dose-dependent Inhibition of ACC by PLP**—ACC preparations that had been partially purified by ammonium sulfate precipitation, were

—80 °C. Alternatively, prior to affinity purification using immobilized monomeric avidin (15, 46), samples were suspended in avidin affinity chromatography buffer, containing 50 mM Tris (pH 7.5), 500 mM KCl, 2 mM EDTA, 2 mM EGTA, 0.02% (w/v) sodium azide. ACC preparations were then incubated with monomeric avidin-agarose for 3 h, the column was washed to remove loosely bound proteins, and biotinylated proteins were eluted with column buffer containing 5 mM biotin. Eluted protein fractions were concentrated to at least 0.2 mg/ml by dialysis against solid polyethylene glycol (Aquacide) or with centrifugal filters.

**Assay of ACC Activity**—ACC was assayed by following the incorporation of \(^{14}\)C from \(^{14}\)C bicarbonate into acid-stable products (15, 46). Briefly, ACC was pre-activated by incubation (30 min, 37 °C) in homogenization buffer containing sodium citrate, as indicated, and bovine serum albumin (2 mg/ml). A 50-μl sample of activated ACC (typically ~1 milliunit), was then added to 450 μl of pre-warmed assay buffer, containing 50 mM HEPES (pH 7.2), 10 mM MgSO\(_4\), 0.5 mM EDTA, 5 mM ATP, 7.5 mM glutathione, 2 mg/ml bovine serum albumin, 150 μM acetyl-CoA, and 7.5 mM potassium \(^{14}\)C bicarbonate (600–1000 dpm/nmol). Assays were terminated by the addition of 200 μl of 2 N HCl, and the mixtures were clarified by microcentrifuge centrifugation and dried under an air stream prior to liquid scintillation counting. To test the effects of PLP, the solid cofactor was first dissolved in 5N HCl, and the solution was then neutralized with 5 N NaOH and diluted as required. One unit of ACC catalyzes the conversion of 1 μmol of acetyl-CoA to malonyl-CoA per min. Kinetic analysis was carried out using SigmaPlot and formulae described previously (47).

**Sodium Borohydride Treatment**—Reduction of aldimine bonds between PLP and protein lysyl residues was carried out using one of two methods. In the first method, ACC preparations were preincubated in the dark (30 min, 25 °C) in the presence of 0.5 mM PLP, followed by the addition of non-radioactive sodium borohydride (1 mM) for a further 15 min at 25 °C. Excess PLP and borohydride were then removed by two wash cycles involving dilution with 10 ml of homogenization buffer, followed by centrifugal concentration at 8,000 \(\times\) g, 4 °C. Controls lacking PLP, borohydride, and citrate were subjected to the same wash cycles. In the second method, sodium \(^{3}\)H borohydride was used, and the samples were subjected to SDS-PAGE to assess \(^{3}\)H incorporation into specific proteins. In this case, ACC was first recovered by ammonium sulfate precipitation and then immobilized by binding to tetrameric avidin (10 mg of avidin per ml of Affi-Gel-10). A sample of ACC (200 μl, containing ~10 μg ACC), was incubated with ~50 μl of avidin beads at 4 °C for 1 h. The beads were then washed 5 times with 1 ml of buffer containing 0.5 mM KCl and finally with water prior to the addition of 0.75 mM PLP. After incubation in the dark (30 min at 25 °C), 1 mM sodium \(^{3}\)H borohydride was added, and the incubation continued for a further 15 min at 25 °C. The beads were extensively washed, as above, and finally boiled in SDS sample buffer.

**SDS-PAGE and Western Blotting**—The discontinuous pH technique (48) was used with 6% mini-gels (w/v acrylamide in separating gel). Electrophoresis was typically carried out for 90 min at 6 mA/gel. Gels were fixed, stained with Coomassie Blue, stabilized with glycerol, and dried between cellulose sheets. Gel lanes were sliced and digested with hydrogen peroxide, and incorporation of \(^{3}\)H into protein bands was determined as described before (49). ACC and other biotin-containing protein subunits were detected with HRP-streptavidin and chemiluminescence reaction following semi-dry blotting as described previously (15, 46).
ity chromatography showed an identical dose-dependence for inhibition (Fig. 1B), confirming that PLP most likely had a direct effect on ACC.

Activation of ACC by incubation with sodium citrate before the addition of PLP led to a significant right shift of the dose-response curve relative to that seen when PLP was added before citrate (Fig. 1C). In contrast, the IC_{50} for inhibition of ACC from skeletal muscle increased only ~20%, to 780 ± 40 μM (mean ± S.E. for three preparations) following citrate treatment.

**Rapid Inhibition of ACC by PLP**—The time dependence for ACC inhibition was first tested by adding PLP (0.5 mM) prior to citrate and subsequent ACC assay (Fig. 2A, filled symbols). The addition of PLP for as little as 1 min led to a substantial inhibition of the subsequent response to citrate. Further incubation with PLP for up to 25 min prior to citrate addition led to a more marked decrease in the response to citrate (Fig. 2B, open symbols).

**FIGURE 3.** Effects of PLP on the substrate kinetics of rat liver ACC. ACC was partially purified from rat liver by ammonium sulfate precipitation and the effects of PLP assessed with respect to bicarbonate (A and B), ATP (C and D), and acetyl-CoA (E and F). ACC was incubated (30 min) in the presence of citrate (10 mM) and in the absence (■) or presence of PLP at 0.05 mM (▲), 0.1 mM (●), 0.25 mM (▲), or 0.5 mM (◆). Assays were carried out with two substrates present at the standard assay concentrations, as indicated under “Experimental Procedures.” The third substrate was added at the concentrations indicated in the primary plots (A, C, and E). Primary and double reciprocal plots (B, D, and F) represent the results of single experiments that were repeated three times with very similar results as summarized in TABLE ONE.
Effects of PLP on substrate kinetics of rat liver ACC

ACC was recovered by ammonium sulfate precipitation from rat liver, and the effects of PLP were assessed with respect to each substrate. Kinetic parameters were derived from data presented in Fig. 3. Values are expressed as mean ± S.E. for three independent experiments with different preparations of ACC. 

| Substrate | PLP | \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) |
|-----------|-----|-----|-----|-------------|
|           | \(\text{mM}\) | \(\text{min}^{-1}\) | \(\text{mM}^{-1}\) | \(\text{min}^{-1} \cdot \text{mM}^{-1}\) |
| Bicarbonate | 0 | 530 | 4.30 ± 0.69 | 123 |
|           | 0.1 | 323 ± 10 | 4.37 ± 0.65 | 74 |
|           | 0.5 | 78 ± 1 | 4.17 ± 0.85 | 19 |
| ATP       | 0 | 530 | 0.63 ± 0.03 | 841 |
|           | 0.1 | 260 ± 9 | 0.21 ± 0.05 | 1238 |
|           | 0.5 | 46 ± 3 | 0.25 ± 0.09 | 184 |
| Acetyl-CoA | 0 | 530 | 0.060 ± 0.014 | 8833 |
|           | 0.1 | 294 ± 32 | 0.063 ± 0.011 | 4667 |
|           | 0.5 | 29 ± 1 | 0.059 ± 0.013 | 492 |

We next tested the effect of direct addition of PLP to the ACC assay mixture. In this experiment, rat liver ACC was first activated by preincubation with 10 mM citrate and then assayed in the absence or presence of the indicated concentrations of PLP (Fig. 2B). In the absence of PLP, the reaction rate was linear for ~10 min and then declined gradually, most likely due to product inhibition by malonyl-CoA, which accumulated to a concentration of 8–16 μM over 10–30 min of assay. The effects of PLP were therefore determined from the initial rates over the first 5 min after PLP addition and only slightly more in the remaining 20 min before addition to the assay.

Effects of PLP on the Substrate Kinetics of ACC—The effects of PLP were assessed with respect to each of the three substrates, using ACC that had been purified through ammonium sulfate precipitation from rat liver. ACC was incubated with the indicated concentrations of PLP (30 min) and then with citrate (20 mM) for 30 min. In the subsequent assay, two substrates were added at the standard assay concentration, as indicated under “Experimental Procedures,” whereas the third substrate varied as indicated (Fig. 3). Results, given as primary and double reciprocal plots, show that, for each variable substrate, PLP led to a mixed or “non-competitive” form of inhibition, with a dominant effect on maximal activity. It is worth noting that double reciprocal plots were linear in the case of acetyl-CoA and bicarbonate but were non-linear in the case of ATP, in agreement with earlier findings of two other groups (50, 51).

Overall, these results rule out competitive inhibition by PLP and also rule out uncompetitive inhibition with respect to acetyl-CoA and bicarbonate. Inhibition constants were derived by plotting 1/v (Dixon plot) or the slope of the double reciprocal lines against the concentration of PLP. The \(K_i\) values were in the range 130–170 μM, regardless of the substrate variable (TABLE ONE).

Effects of PLP on ACC Activation by Citrate—Results presented in Figs. 1 and 2 demonstrated that responses of ACC to PLP were influenced by prior addition of citrate. The interactions between citrate and PLP were therefore investigated in more detail (Figs. 4 and 5). ACC activity was reduced by PLP in a dose-dependent manner at all concentrations of citrate. The inhibition was complex, with a marked decline in maximal activity and a substantial increase in \(K_m\), values being the average of two experiments as described in Fig. 2B. Effects of PLP in the assay were achieved in the continued presence of 1 mM citrate, carried over from the preincubation.

Effects of PLP Interacts Reversibly with Lysyl Residue(s) of ACC—When rat liver ACC was incubated with citrate prior to addition of PLP (Fig. 5), the \(K_i\) for citrate activation increased from ~2 mM in the absence of PLP to >10 mM in the presence of 0.5 mM PLP (Fig. 4 and TABLE TWO). From double-reciprocal plots (Fig. 4B), as well as Dixon plots, the derived \(K_i\) value was close to 100 μM (TABLE TWO). These plots were linear at high concentrations of citrate but deviated at lower citrate levels, reminiscent of the double reciprocal plots seen with ATP (Fig. 3).

Similar kinetic analyses were carried out in which ACC was incubated with citrate prior to addition of PLP (Fig. 5), the \(K_i\) in this case being ~4-fold higher than the value observed following addition of PLP prior to citrate (TABLE TWO). Interestingly, in similar experiments with ACC from rat skeletal muscle, the \(K_i\) for inhibition by PLP was approximately the same whether PLP was added before or after citrate, the values being ~350 μM in both cases.

PLP Interacts Reversibly with Lysyl Residue(s) of ACC—When rat liver ACC was incubated first with 0.5 mM PLP and then subsequently with
20 mM citrate, the substantial inhibition observed (~80%) was significantly reversed by de-salting to remove PLP while maintaining the concentration of citrate (Fig. 6). In contrast, de-salting did not reverse the inhibitory effect observed following sequential treatment with PLP and sodium borohydride (Fig. 6). Addition of 1 mM sodium borohydride alone had no effect on the subsequent activation of ACC with 20 mM citrate. These results are consistent with the possibility that a lysyl side chain of ACC reacts with PLP to form an aldimine that can be reduced with sodium borohydride.

To further test the nature of the effect of PLP, we attempted to reverse ACC inhibition with hydroxylamine and amino-oxyacetate, reagents that are known to react with free or protein-bound PLP (52, 53). Neither hydroxylamine nor amino-oxyacetate had any detectable effect on ACC activity when added prior to citrate activation (Fig. 7). Based on the results of eight separate experiments with different ACC preparations, only the ACC bands consistently showed 3H labeling above the background level (Fig. 8A). The incorporation of 3H into ACC subunits was dose-dependent with respect to PLP concentration, with no significant 3H incorporation observed if avidin-immobilized proteins were treated with sodium [3H]borohydride in the absence of PLP. The gel slices containing subunits of pyruvate carboxylase or of the other biotin-dependent carboxylases that had bound to the immobilized avidin did not contain any detectable 3H label above the background. The molar incorporation of 3H into ACC subunits was estimated using the specific activity of the applied sodium [3H]borohydride, the activity of ACC applied to the beads and assuming a specific enzyme activity of 1.5 unit/mg of protein, a value typical of many preparations.

![Diagram](image)

**FIGURE 5.** Dose-dependent effects of PLP following preincubation of ACC with citrate. The experimental design and symbols are as described in the legend to Fig. 4, except that ACC was first incubated for 30 min with the indicated concentrations of citrate and then further incubated in the absence or presence of the indicated concentrations of PLP. Results are from one experiment that was repeated with very similar results, summarized in TABLE TWO.

![Diagram](image)

**FIGURE 6.** Effects of desalting and borohydride reduction on PLP-treated ACC. Samples of liver ACC, partially purified by ammonium sulfate precipitation, were incubated for 30 min with no further additions (lane 1), 1 mM sodium borohydride (lane 2), or 0.5 mM PLP (lane 3), prior to citrate activation (20 mM for 30 min) and assay. Alternatively, ACC was treated with PLP as above and then desalted in the presence of citrate prior to assay (lane 4). Finally, PLP-treated ACC was incubated with 1 mM sodium borohydride prior to desalting in the presence of citrate and assay (lane 5). ACC activities are expressed relative to the maximum with 20 mM citrate and calculated as mean ± S.E. for n = 3–6 independent experiments. The maximum activities of ACC used in these experiments was in the range 250–300 milliunits/ml.

### TABLE TWO

| PLP (mM) | Citrate activity (% maximum) |
|---------|-----------------------------|
| 0       | 98 ± 15                     |
| 0.1     | 2.8 ± 0.9                   |
| 0.5     | 39.3 ± 36.2                 |

*ND, not determined.*

3H incorporation into each slice was determined. The location of the subunits of ACC (280 and 265 kDa) and other biotinylated proteins, principally pyruvate carboxylase (120 kDa), was confirmed by Western blotting with HRP-streptavidin. Based on the results of eight separate experiments with different ACC preparations, only the ACC bands consistently showed 3H labeling above the background level (Fig. 8A). The incorporation of 3H into ACC subunits was dose-dependent with respect to PLP concentration, with no significant 3H incorporation observed if avidin-immobilized proteins were treated with sodium [3H]borohydride in the absence of PLP. The gel slices containing subunits of pyruvate carboxylase or of the other biotin-dependent carboxylases that had bound to the immobilized avidin did not contain any detectable 3H label above the background. The molar incorporation of 3H into ACC subunits was estimated using the specific activity of the applied sodium [3H]borohydride, the activity of ACC applied to the beads and assuming a specific enzyme activity of 1.5 unit/mg of protein, a value typical of many preparations.
PLP leads to a dose-dependent but is not yet conclusive. In support of this contention, prior exposure to citrate. The evidence so far obtained is consistent with this possibility of the three substrates and with respect to the allosteric activator citrate. Our initial hypothesis was that PLP would bind to ACC, as it does to phosphofructo-1-kinase and carboxylate carriers, at the same site as the normal concentrations of the substrates. Taken together, these observations suggest that the effects of PLP are more likely to occur in the solid-state approach used so far, in which ACC is immobilized to avidin-agarose and conditions were designed to optimize for tetrameric avidin-agarose beads. The beads were stringently washed and then incubated with PLP and [3H]borohydride. Proteins were eluted by boiling in SDS sample buffer and subjected to SDS-PAGE. Gels were sliced, and [3H]incorporation was determined as shown (14). Gel slices (5 mm) are numbered from origin to tracking dye (14). Numbered arrows indicate the migration of ACC subunits, 280-kDa and 265-kDa (1), myosin, 220-kDa (2), phosphorylase a, 98-kDa (3), and serum albumin, 68-kDa (4). Results are given as the mean ± S.E. for six independent experiments with different ACC preparations from rat liver. In 8, incubations were carried out with the indicated concentrations of PLP, but otherwise identical conditions.

DISCUSSION

In the studies reported here, we have demonstrated that PLP is a linear and reversible inhibitor of ACC-1 and ACC-2 from rat liver, adipose, and muscle tissues. Highly purified ACC is equally sensitive to PLP, suggesting the interaction is direct and not mediated by another protein. The inhibition of ACC by PLP is complex with respect to each of the three substrates and with respect to the allosteric activator citrate. Our initial hypothesis was that PLP would bind to ACC, as it does to phosphofructo-1-kinase and carboxylate carriers, at the same site as citrate. The evidence so far obtained is consistent with this possibility but is not yet conclusive. In support of this contention, prior exposure to PLP leads to a dose-dependent -4-fold increase in the $K_i$ for ACC activation by citrate. Conversely, pre-exposure to citrate reduces the sensitivity of rat liver ACC to PLP, the $K_i$ being increased >3-fold. In contrast to the evident interactions between PLP and citrate, ACC inhibition was not associated with any substantial change in substrate $K_m$ values and was not diminished in assays containing two to three times the normal concentrations of the substrates. Taken together, these observations suggest that the effects of PLP are more likely to occur through interaction at or near the citrate-binding site than at the sites for substrate binding. An important caveat is that we have not been able to carry out competitive binding experiments to confirm that the binding of citrate and PLP are mutually exclusive. Because the $K_i$ is in the millimolar range, citrate binding experiments will require large quantities of ACC that are very difficult to obtain from primary rat tissues. In principle, the [3H]labeling studies might reveal citrate protection. However, in the solid-state approach used so far, in which ACC is immobilized to avidin-agarose and conditions were designed to optimize for PLP interactions, the presence of citrate did not diminish [3H]incorporation in the presence of high concentrations of PLP.

PLP forms a reversible Schiff base (aldimine) with the ε-amino groups of lysyl residues in the active sites of a number of PLP-dependent
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Inhibitory effects of PLP and related compounds on rat liver ACC

| Compound                  | Main structural features | Inhibition (%) |
|---------------------------|--------------------------|----------------|
| Pyridoxal phosphate       | PyR OH CHO CH$_2$OP COOH | 91.5           |
| Pyridoxal                 | + + + - + - - - - - - - | 50.7           |
| Pyridoxine                | + + + - + - + + + + + + | 6.0            |
| 4-Pyridoxic acid          | + + + + + + + + + + + + | 16.4           |
| Pyridoxamine phosphate    | + + + + + + + + + + + + | 16.3           |
| Pyridoxamine              | + + + + + + + + + + + + | + + + + + + + + | 20.7           |
| Phosphofructo-1-kinase     | + + + + + + + + + + + + | 3.3            |
| 3,5-Diacetylcarboxylic acid| + + + + + + + + + + + + | 79.9           |
| Nicotinamide              | + + + + + + + + + + + + | 1 23           |
| 1,3-acetol nicotinamide   | + + + + + + + + + + + + | 2 3.4          |

Enzymes and appears to interact similarly at the citrate binding sites of phosphofructo-1-kinase and mitochondrial carboxylate carriers. Several observations support the concept that PLP also inhibits ACC via a Schiff base reaction. For example, removing PLP through washing or by reaction with alternative amino reagents such as hydroxylamine or amino-oxycetate reversed the inhibition of ACC. In contrast, the inhibition of ACC was no longer reversible if the Schiff base was reduced with sodium borohydride. Furthermore, stringent purification by avidin affinity chromatography followed by SDS-PAGE revealed that ACC subunits were directly labeled when incubated with PLP plus sodium $[^3]$H]borohydride. Although PLP is able to interact with many different proteins, it still does so in a selective manner at specific sites within the target proteins. This is also true in the case of ACC, the stoichiometry of labeling with PLP plus $[^3]$H]borohydride being $<$1 mol/mol ACC subunit despite the presence of $>$100 lysyl residues in each subunit. Based on these observations, we conclude that PLP binds directly to ACC subunits and that the interaction occurs at specific lysyl residues that are located within a particular three-dimensional context. The specificity of the interaction of PLP with ACC is further underlined by the fact that the effects were observed in the presence of excess serum albumin and by the observation that related biotinylated proteins, including pyruvate carboxylase, and other mitochondrial carboxylases were not labeled in the presence of PLP plus sodium $[^3]$H]borohydride, despite the common features of their respective reaction mechanisms and active sites.

It is anticipated that the results presented here will provide the basis to begin to map the specific lysyl residues of ACC that are required for binding of PLP and, by inference, of citrate. However, labeling of ACC with PLP will likely provide only one step toward the definition of the complex citrate-binding site. Based on the work done with phosphofructo-1-kinase, for example, crystallographic studies have shown that the residues required for citrate binding are widely separated within the primary sequence, notably residues Arg-21, Arg-25, Arg-54, Ser-58, Asp-59, Arg-154, Gly-185, Glu-187, Lys-213, Lys-214, His-215, and Tyr-319 of the Escherichia coli enzyme, indicating that the site will only be fully appreciated with knowledge of the three-dimensional structure of the protein (54, 55). So far, no high resolution structure of any citrate-sensitive ACC has been determined, and this goal will not likely be realized until heterologous expression can be achieved.

Defining the location of the citrate-binding site and the relationship of this site to the active sites and other allosteric sites of ACC will be challenging. With all allosteric proteins studied so far, the regulatory ligand-binding sites are significantly removed from the respective active sites; typically by 30–40 Å (56). Considering that ACC does not display typical allosteric sigmoid substrate kinetics and that citrate influences all partial reactions (forward and reverse), it is possible that citrate binding occurs closer to the active centers than is the case for other allosteric proteins. To further complicate matters, it is possible that acetyl-CoA and ATP may bind at regulatory as well as active sites on ACC. A comprehensive analysis of ligand binding, kinetic, and structural studies will ultimately be required to define all the relevant sites.

The estimated $K_i$ for inhibition of ACC by PLP at low citrate concentrations is similar to the concentrations of PLP (100–200 μM) measured in rat liver (57). However, a large proportion of the total pool of enzyme is bound to PLP-dependent enzymes, some of which have much greater affinity for the coenzyme (58). It is therefore improbable that PLP is a physiologically relevant ACC inhibitor. The actions of PLP on ACC are nevertheless important for several reasons. For example, the actions of PLP suggest that structurally related natural coenzymes or metabolites might play a role in controlling ACC activity as endogenous inhibitors. For example, metabolites of tryptophan are potent ACC inhibitors, although they appear to act by a different mechanism, probably involving effects at the active site (5). In addition, the key features of the PLP structure highlighted here may provide the basis for rational design of further ACC inhibitors. Recent reports, sparked by the phenotype of mice lacking ACC-2 (59, 60) suggest that the development of ACC inhibitors could have considerable potential in the treatment of obesity, insulin resistance, and type 2 diabetes. Among effective inhibitors developed recently, most seem to be directed at the ACC active site (61, 62). Because ACC is unique among biotin-dependent carboxylases in sensitivity to citrate, targeting this allosteric site may have selective advantages.

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