Regulation of Acetyl-CoA Carboxylase by Guanine Nucleotides*

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Acetyl-CoA carboxylase, a major rate-limiting enzyme for fatty acid synthesis, is subject to acute regulation by both allosteric modulation and covalent enzyme phosphorylation. Because citrate activation of the enzyme in vitro requires citrate concentrations far in excess of intracellular levels, we have attempted to identify other ligands which might mediate carboxylase activity. Heated liver extracts contain a potent endogenous activator of carboxylase assayed in dialyzed high speed liver supernatant; the activator elutes behind the salt volume of a Bio-Gel P-6 gel filtration column, is destroyed by alkaline phosphatase, and is adsorbed by charcoal. This activator activity is shared by several guanine nucleotides (5'-GTP, 5'-GDP, 5'-GMP, and 3':5'-cyclic GMP). Further separation of the endogenous activator by high pressure liquid chromatography reveals a carboxylase-activating compound which co-elutes with 5'-GMP. The guanine nucleotides are potent activators of carboxylase activity at intracellular nucleotide concentrations and permit expression of maximal enzyme velocity at cytosolic citrate concentrations. However, we have been unable to document any effects of guanine nucleotides on isolated rat liver acetyl-CoA carboxylase. While the mechanisms of these effects remain to be elucidated, they suggest that the guanine nucleotides may be important intracellular regulators of carboxylase activity and of fatty acid synthesis.

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**EXPERIMENTAL PROCEDURES**

Materials—Male C.D. rats were obtained from Charles River Breeding Laboratories. NaH14CO3 was obtained from New England Nuclear. Acetyl-CoA was synthesized by the method of Simon and Shemin (5). L-1-Tosylamido-2-phenylethyl chloromethyl ketone trypsin, pancreatic trypsin, and pronase were purchased from Worthington. Bio-Gel P-6 was obtained from Bio-Rad Laboratories.

Acetyl-CoA carboxylase was prepared from the livers of rats who had been fasted for 72 h and refed for 48 h with a low fat, high carbohydrate diet (Fat Free Rat Diet, Bioserv, Inc.). Rats were killed by cervical dislocation, and livers were rapidly removed and homogenized (1 g/3 ml) in Tris.Cl (10 mM, pH 7.20), dithiothreitol (2 mM), and sucrose (0.25 M) at 4 °C in a glass homogenization vessel with five strokes of a motor-driven Teflon pestle. The homogenate was centrifuged at 20,000 × g for 20 min in a Sorvall RC2 centrifuge. This supernatant was then centrifuged at 105,000 × g for 60 min in a Beckman L2 ultracentrifuge. The high speed supernatant was filtered through glass wool and dialyzed overnight against 500 volumes of Tris-Cl (10 mM, pH 7.2) and dithiothreitol (2 mM). After dialysis, aliquots were frozen at −20 °C; enzyme activity remained stable for at least one week under these conditions.

Acetyl-CoA carboxylase was assayed by a previously published technique (16) with slight modification. In the routine assay for enzyme activators, activator fractions were added to tubes containing assay buffer, MgCl2 (5 mM), and citrate (0.2 mM) at 4 °C. Acetyl-CoA, ATP, and NaH14CO3 were added just prior to assay. The tube was prewarmed for 20 s at 37 °C, and the reaction was initiated by the addition of dialyzed liver supernatant. The routine assay was terminated after 2 min by the addition of 10% perchloric acid and products analyzed as previously reported (16). Blank assays (−acetyl-CoA) were performed for each individual assay. Acetyl-CoA carboxylase activity is expressed (except as indicated) as milliunits per mg of protein, where 1 milliunit equals 1 nmol of C02 fixed to malonyl-CoA per min.

Liver extracts were prepared by homogenizing livers (1 g/2 ml) from fasted/refed rats with a Waring Blender at top speed for 1 min in Tris-Cl (10 mM, pH 7.2) and dithiothreitol (2 mM) at 23 °C. The homogenate was then heated at 75–80 °C for 5 min in a boiling water bath and cooled on ice. The heated extract was then centrifuged at 20,000 × g for 20 min. This supernatant (up to 70 ml) was applied to a Bio-Gel P-6 gel column (2.5 × 90 cm) equilibrated with Tris-Cl (10 mM, pH 7.2) eluting at a flow rate of 0.5–0.7 ml/min. Fractions beyond the void volume (identified as the major A280 peak) were assayed in the activator assay, as above.

HPLC of endogenous carboxylase activator fractions and guanine nucleotides was performed with a Beckman/Altex model 334 MP

The abbreviations used are: GMP-PNP, guanylyl imidodiphosphate; HPLC, high pressure liquid chromatography.
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instrument equipped with a Hitachi UV/Via variable wave length detector. Separations were carried out with a Whatman Partisil SAX-10 column (250 × 6.35 mm outer diameter) following the general protocol of Hartwick and Brown (17) with some modifications. After sample injection, compounds were eluted isocratically in potassium phosphate (7 mM, pH 4.0) for 35 min followed by a gradient to 100% potassium phosphate (0.25 M, pH 4.5) with potassium chloride (0.5 M) at 1% per min. Column operating parameters were: flow rate, 1 ml/min, column pressure, 800–1000 p.s.i., and ambient room temperature. Sample injection volumes varied between 100 μl and 2000 μl in individual experiments. At least 20 min were allowed for column re-equilibration after each run. Deionized water that had been filtered through a 0.45-μ filter (Millipore) and reagent grade potassium phosphate and potassium chloride (Mallinckrodt) were used to prepare the buffers, which were adjusted to pH with either phosphoric acid or potassium hydroxide and refiltered prior to use. Detection of eluting compounds was at 254 nm coupled with assay of individual fractions in carboxylase activator assay.

Rat liver acetyl-CoA carboxylase was isolated from the livers of fasted/refed rats by avidin affinity chromatography, which is a modification of the general technique of Gravel et al. (18). This technique yields enzyme which has a specific activity of 1.5–2.0 units/mg of protein and displays a single band of molecular weight 240,000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Protein determination was by the method of Lowry et al. (19) employing bovine serum albumin as a standard.

RESULTS

Dialysis of a 105,000 × g supernatant prepared from the livers of fasted/refed rats resulted in a marked alteration of the acetyl-CoA carboxylase citrate dose-response curve (Fig. 1). The velocity at low citrate concentrations falls dramatically (5- to 6-fold) with dialysis, while V_max at 5 mM citrate is only diminished by 2-fold. The data does not lend itself to analysis by Michaelis-Menten kinetics; it can be noted, however, that the half-maximally activating citrate concentration rises from approximately 0.4 mM to 1.25 mM with dialysis of the supernatant. These data suggested that dialyzable low molecular weight ligands might be permissive in the expression of maximal enzyme velocity at low citrate concentrations.

A heated (75 °C, 5 min) extract was prepared from the same livers and applied to a P-6 gel filtration column. Each fraction beyond the void volume material (as identified by A_280) was assayed in the activator assay, using dialyzed supernatant enzyme as a substrate. As shown in Fig. 2, there is a peak of carboxylase activator activity identified behind the salt volume (Ve = 460 ml). We were unable to detect this material in the assay of the unfractionated extract. The reason for this became apparent with fractionation; the salt volume (V_s) contains unidentified ligands that markedly increase the activity of the blank reaction (−acetyl-CoA).

Preliminary characterization of the activator fraction (pooled from the peak eluting behind the salt volume) is shown in Table I. The activity of this material is adsorbed by E. coli alkaline phosphatase. Activity was preserved after incubation with trypsin, chymotrypsin, and pronase. These data suggested that the material might represent a nucleotide. Accordingly, we screened a large number of various compounds for similar activator activity.

Only the guanine nucleotides with a 5'-phosphate group were found to possess carboxylase activator activity (Table IIA). No activation of enzyme activity was found with ADP, AMP, 3'-5'-cyclic AMP, UTP, UMP, CTP, CMP, 3'-5'cCMP, ITP, IMP, 3'-5'-cyclic IMP, XTP, XDP, XMP, or 5'-cyclic XMP when measured at 100 μM and 1 mM (not shown); guanosine, ribose-5-phosphate, 2'-GMP, 3'-GMP, 2',3'-cyclic GMP, and the nonhydrolyzable GTP analog, GMP-PNP, did not lead to enzyme activation (Table IIA).  

Fig. 1. Acetyl-CoA carboxylase citrate dose-response in undialyzed and dialyzed liver supernatants. Acetyl-CoA carboxylase activity was measured at varying citrate concentrations between 0 and 5 mM in undialyzed 105,000 × g liver supernatant (see under "Methods") (●) and supernatant which had been dialyzed overnight against 500 volumes of Tris-Cl (10 mM, pH 7.2) and dithiothreitol (2 mM) (□). Blank reactions (−acetyl-CoA) were also performed at each citrate concentration and have been subtracted from the displayed data.

Fig. 2. Fractionation of endogenous carboxylase activator. A representative fractionation (n = 0) is shown. Heated extract of liver homogenate was prepared and fractionated on a Bio-Gel P-6 column as described under "Methods." Each fraction beyond the void volume was assayed for carboxylase activator activity after addition to a dialyzed 105,000 × g supernatant, prepared as detailed. The fixation of H^14CO_3^- was determined both in the presence (●) and absence (blank reaction) (○) of acetyl-CoA. The results of both assays are shown plotted as nanomoles of H^14CO_3^- fixed per min. V_s indicates the salt volume and V_e the elution volume of the carboxylase activator.

Given these observations, the endogenous activator from the P-6 column was further fractionated by HPLC in the anion exchange mode (see under "Methods"), employing a
TABLE I
Preliminary characterization of endogenous activator

Dialyzed supernatant enzyme was assayed at 0.2 mM citrate (see under "Methods") in the presence of no additions or an activator fraction obtained from the P-6 column (see Fig. 2). This endogenous activator was treated in turn with E. coli alkaline phosphatase (1.3 units/ml), pronase (10 µg/ml), trypsin (10 µg/ml), or chymotrypsin (20 µg/ml) for 20 min. The endogenous activator was also adsorbed by acid-washed charcoal (20 mg/ml) for 20 min. For each condition, a blank reaction (-endogenous activator) was run; also for each carboxylase assay, a minus acetyl-coA blank assay was performed.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Carboxylase activity (milliunits/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.21</td>
</tr>
<tr>
<td>Endogenous activator</td>
<td>1.10</td>
</tr>
<tr>
<td>+ Alkaline phosphatase</td>
<td>0.25</td>
</tr>
<tr>
<td>+ Charcoal adsorption</td>
<td>0.21</td>
</tr>
<tr>
<td>+ Pronase</td>
<td>1.09</td>
</tr>
<tr>
<td>+ Trypsin</td>
<td>1.06</td>
</tr>
<tr>
<td>+ Chymotrypsin</td>
<td>0.92</td>
</tr>
</tbody>
</table>

TABLE II
Activation of acetyl-CoA carboxylase by guanine nucleotides

Dialyzed supernatant enzyme was assayed at 3.2 mM citrate (see under "Methods") in the presence of no additions or various guanine nucleotides at 50 µM final concentration in A. In B, the endogenous activator (85 µM peak fraction from the P-6 column) or guanine nucleotide (GTP, 300 µM; GDP, 100 µM; 5'-GMP, 30 µM) alone or in varying concentrations were employed.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Carboxylase activity (milliunits/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td>0.20</td>
</tr>
<tr>
<td>5'-GTP</td>
<td>1.03</td>
</tr>
<tr>
<td>5'-GMP</td>
<td>0.95</td>
</tr>
<tr>
<td>5'-GDP</td>
<td>0.91</td>
</tr>
<tr>
<td>3'-5'-cGMP</td>
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</tr>
<tr>
<td>2'-GMP</td>
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</tr>
<tr>
<td>3'-GMP</td>
<td>0.20</td>
</tr>
<tr>
<td>2'-3'-cGMP</td>
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</tr>
<tr>
<td>GMP-PNP</td>
<td>0.19</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.02</td>
</tr>
<tr>
<td>Ribose-5-P</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Addition</th>
<th>Carboxylase activity (milliunits/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. Endogenous activator</td>
<td>0.48</td>
</tr>
<tr>
<td>Endogenous activator + 5'-GTP</td>
<td>1.01</td>
</tr>
<tr>
<td>5'-GTP + 5'-GDP + 5'-GMP</td>
<td>1.16</td>
</tr>
<tr>
<td>5'-GTP + 5'-GDP</td>
<td>1.18</td>
</tr>
<tr>
<td>5'-GTP + 5'-GMP</td>
<td>1.14</td>
</tr>
<tr>
<td>5'-GDP + 5'-GMP</td>
<td>1.16</td>
</tr>
</tbody>
</table>

No peaks corresponding to 5'-GMP or 5'-GDP are observed. Some preparations have shown a small peak eluting with a retention time similar to 3'-5'-cyclic GMP, but this has not been a consistent observation.

This same column was employed to determine the purity of the guanine nucleotides tested; the results indicated that 3'-5'-cyclic GMP, 5'-GTP, 5'-GDP, and 5'-GMP were not contaminated with each other; >99% of the UV-absorbing material in each was present in a single peak (not shown). Furthermore, seven different preparations of 5'-GTP from three sources all displayed equipotent activator activity (not shown).

We have attempted to establish further whether endogenous 5'-GMP represents all of the activator material isolated from the P-6 column. As stated above, there is a peak with a retention time identical with that of pure GMP; mixing experiments with 5'-GMP and the endogenous activator fraction with subsequent HPLC fractionation showed the expected augmentation of this peak (not shown). This peak was isolated after HPLC fractionation of both 5'-GMP and the endogenous activator fraction. As shown in the insets to Fig. 3, B and C, activator activity was recovered only from this peak in both commercial 5'-GMP and the endogenous activator.

However, we regard this as only tentative evidence that 5'-GMP is the sole endogenous activator present in the extract, because of the possible limitations of assay sensitivity and because higher salt concentrations above the GMP peak further into the phosphate/chloride gradient severely inhibit acetyl-CoA carboxylase activity. A further argument that 5'-GMP may be the only endogenous activator isolated under these conditions is presented below.

Dose-response curves to each of the four guanine nucleotides were performed (Fig. 4). 5'-GTP and 5'-GDP showed identical curves with a half-maximally activating concentration of approximately 25 µM; 5'-GMP is less potent at concentrations >30 µM, and in fact, the activation diminishes at concentrations >100 µM. The 3'-5'-cyclic GMP curve is shifted to the right with a much diminished maximal activator. When assayed in combination with each other or with the endogenous activator, no augmentation or diminution of activator activity was observed (Table II B). Row 3 in this table shows that at prevailing intracellular guanine nucleotide concentrations (21, 22), maximal activator activity is observed.

3 There is also elution coincidence of the 5'-GMP peak and a major peak in the endogenous activator in a second HPLC system. These fractions (not shown) were performed with a Aminex A-27 column (Bio-Rad) eluting isocratically with NaP04, 0.01 M, NaCl, citrate, 0.05 M, and ethanol (28.5%, v/v) at pH 8.35. This buffer system does not permit detection of activator activity in unfraccionated or fractionated material.
The 5'-GMP dose-response curve was employed in conjunction with HPLC fractionation to further test the hypothesis that 5'-GMP was the sole endogenous activator. A linear standard curve relating peak area (A<sub>405</sub>) to nanomoles of 5'-GMP injected into the HPLC column was constructed (Fig. 5). The peak area corresponding to the co-eluting peak in three preparations of endogenous activator was then determined by the same technique, and the activating potency of each endogenous preparation was determined with the standard activator assay. It was then assumed that all of the stimulating activity in the endogenous preparation was due to 5'-GMP, and thus the 5'-GMP content in the endogenous preparation could be determined from the GMP dose-response curve shown in Fig. 4. If this assumption were correct, the data points generated from analysis of the endogenous activator should fall on the 5'-GMP standard curve determined by HPLC. As indicated in Fig. 5, close correspondence is observed with three separate preparations. This further strengthens the hypothesis that 5'-GMP is the sole endogenous activator in our preparation.

The effects of the guanine nucleotides are most strikingly observed in the time course of the acetyl-CoA carboxylase catalytic reaction. Fig. 6 shows that there is an initial hysteresis both in the presence and absence of 5'-GTP and that the reaction is linear thereafter up to about 3 min. The initial velocity calculated from the linear portion of this curve is 7-fold higher in the presence of 100 μM 5'-GTP. Similar results were obtained for 5'-GMP and the endogenous activator (not shown).<sup>5</sup>

<sup>5</sup>5'-GMP also elutes behind the salt volume of a Bio-Gel P-6 column. Of interest was the observation that 5'-GTP eluted in an identical fraction, yet no GTP was observed in the endogenous preparation (by HPLC). When [γ<sup>32</sup>P]GTP was added to the liver homogenate prior to heating and subsequently fractionated, all the γ<sup>32</sup>P appeared in the salt volume, and no GTP could be detected in this fraction by HPLC. Thus, the γ-phosphate appears to be hydrolyzed during preparation and subsequent fractionation.<sup>6</sup>

One potential explanation for alteration of apparent carboxylase activity is that the guanine nucleotides inhibit malonyl-CoA decarboxylase, thus preserving to a greater extent the measured product of the carboxylase reaction. Preliminary experiments indicate that the guanine nucleotides have no effect on malonyl-CoA decarboxylase activity (not shown).

The effects of the guanine nucleotides are potent stimulators of acetyl-CoA carboxylase activity as measured in dialyzed liver supernatants. Furthermore, we have identified an endogenous activator in heated liver extracts and have tentatively identified it as guanosine 5'-monophosphate. The effects of the guanine nucleotides (at intracellular concentrations) are particularly striking, when viewed in concert with the carboxylase citrate dose-response curve. The addition of nucleotide permits expression of maximal enzyme velocity at known prevailing cytosolic citrate concentrations (1, 12–14), while enzyme assayed in the absence of nucleotide does not attain V<sub>max</sub> until ≥4 mM citrate. It is of interest that many investigators have assayed supernatant enzyme after removing small molecules by desalting procedures or prior ammonium sulfate precipitation (9–11, 20); these procedures would remove guanine nucleotides and would, therefore, markedly alter apparent enzyme activity.

**DISCUSSION**

The results of the present study indicate that guanine nucleotides are potent stimulators of acetyl-CoA carboxylase activity as measured in dialyzed liver supernatants. Furthermore, we have identified an endogenous activator in heated liver extracts and have tentatively identified it as guanosine 5'-monophosphate. The effects of the guanine nucleotides (at intracellular concentrations) are particularly striking, when viewed in concert with the carboxylase citrate dose-response curve. The addition of nucleotide permits expression of maximal enzyme velocity at known prevailing cytosolic citrate concentrations (1, 12–14), while enzyme assayed in the absence of nucleotide does not attain V<sub>max</sub> until ≥4 mM citrate. It is of interest that many investigators have assayed supernatant enzyme after removing small molecules by desalting procedures or prior ammonium sulfate precipitation (9–11, 20); these procedures would remove guanine nucleotides and would, therefore, markedly alter apparent enzyme activity.
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Fig. 6. Time course of 5′-GTP activation of acetyl-CoA carboxylase. Acetyl-CoA carboxylase activity in a dialyzed 105,000 × g supernatant in the presence (Δ—Δ) and absence (○—○) of 5′-GTP (100 μM) was determined at 30 °C over time sampling every 15 s to 3 min and every 30 s thereafter. Blank reactions (−acetyl-CoA) have been subtracted from each data point. Similar data has also been obtained for 5′-GMP and the endogenous activator (not shown). Carboxylase activity is expressed as milliunits per mg of protein, where 1 milliunit equals 1 nmol of H14CO3− fixed.

Fig. 7. Acetyl-CoA carboxylase citrate dose-response curve in the presence and absence of 5′-GMP. Citrate dose-response curves for carboxylase activity were performed assaying dialyzed 105,000 × g supernatant enzyme in the presence (●—●) and absence (○—○) of 5′-GMP, (100 μM) as in Fig. 1. Identical results were obtained for 5′-GTP (not shown).

and citrate reactivity.

We do not feel that the present data permit identification of the intracellular guanine nucleotide that might serve as the carboxylase regulator. 5′-GTP, 5′-GDP, and 5′-GMP are all potent activators at known intracellular concentrations (21, 22). While the endogenous activator preparation contains only 5′-GMP, it is likely that hydrolysis of 5′-GTP and perhaps 5′-GDP occurs under the conditions of activator preparation. The failure of GMP-PNP to activate carboxylase suggests that 5′-GTP hydrolysis might be required for expression of the 5′-GTP effects. Until further insight into the mechanism(s) of these effects is gained, speculation is unwarranted.

Fig. 8. Effects of 5′-GTP and endogenous activator on dialyzed and undialyzed liver supernatant. Acetyl-CoA carboxylase activity was determined in both dialyzed and undialyzed 105,000 × g supernatants in the absence (light stippled bars) and presence of 5′-GTP (hatched bars) or the endogenous activator (heavy stippled bars). Blank reactions (−acetyl-CoA) were performed under each condition and have been subtracted from the displayed data.

Fig. 9. Isolated rat liver acetyl-CoA carboxylase and guanine nucleotides. Isolated rat liver acetyl-CoA carboxylase (see under “Methods”) was assayed at 0.2 mM citrate over a time course (see Fig. 6) in the absence (○) and presence of 5′-GTP (●) or the endogenous activator (△). The failure of guanine nucleotide to activate purified enzyme has also been observed with 5′-GDP, 5′-GMP, and 3′5′-cyclic GMP (not shown).

We have consistently been unable to observe any effects of guanine nucleotides on isolated rat liver enzyme, in contrast to their potent effects on enzyme in the dialyzed high speed supernatant fraction. It is possible that alteration(s) of carboxylase during isolation might render it insensitive to the action of guanine nucleotides. Alternatively, additional factors may be required for the expression of nucleotide sensitivity.
Work in progress is aimed at testing both these possibilities. We would emphasize that there may be other endogenous regulators of carboxylase activity that either elude our assay detection or are not stable to heating. Although the addition of guanine nucleotides to dialyzed supernatant largely reverses the alteration in carboxylase activity that occurs with dialysis, one cannot conclude that these are the only dialyzable carboxylase activators. Furthermore, we cannot exclude the possibility that our HPLC fractions contain additional compounds that co-elute with guanosine 5'-monophosphate and that are also present in commercially prepared guanine nucleotides. Both coenzyme A (23) and DL-palmitoyl carnitine (24) have been reported to be carboxylase activators; neither compound, in our hands, reproduces the effects of the guanine nucleotides and the endogenous activator (not shown).

Note Added in Proof—After submission of this article, additional evidence for the co-identity of the endogenous activator and 5'-GMP has been gained. Isolated endogenous activator has a molecular weight identical with that of 5'-GMP, as determined by fast atom bombardment mass spectrometry, and a fragmentation spectra similar to 5'-GMP by direct ionization. These determinations were kindly performed by Dr. Vernon Reinhold of Harvard Medical School.

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REFERENCES