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A MALONYL-CoA-BINDING PROTEIN FROM LIVER

Richard E. Dugan,[1,2]* Bengt R. Osterlund,[3] Roger F. Drong,[3] and Theresa L. Swenson[3]

[1]Metabolic and Lipid Research Laboratory, William S. Middleton Memorial Veterans Hospital, Madison, WI

Departments of [2]Neurology and [3]Physiological Chemistry, University of Wisconsin Medical School, Madison, WI

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Summary: A soluble protein that binds malonyl-CoA without requiring cofactors has been purified from rat liver. Until saturated, it competes with fatty acid synthetase for free malonyl-CoA, temporarily reducing the rate of fatty acid synthesis at low levels of malonyl-CoA, as in fatty acid synthetase-coupled assays for acetyl-CoA carboxylase. These assays yield low estimates for carboxylase activity with crude and partially purified homogenates containing the malonyl-CoA-binding protein. The protein does not inhibit assays for carboxylase activity that measure nonvolatile radioactivity incorporated from bicarbonate or NADH oxidation coupled to ADP formation. It has an M_r of 180,000 and a subunit of 90,000. It has a lower affinity for ATP, ADP, and acetyl-CoA and none for CO_2 or fatty acid synthetase. No enzymatic function has been identified. The protein may regulate malonyl-CoA-binding enzymes.

In assays for acetyl-CoA carboxylase activity that are coupled to fatty acid synthesis, a steady state is attained in which the velocity of malonyl-CoA synthesis equals the velocity of its incorporation into fatty acids. Conditions are adjusted so that the steady-state concentration of free malonyl-CoA approaches zero, and the level of fatty acid synthetase-bound malonyl-CoA and the rate of fatty acid synthesis are constant and independent of added fatty acid synthetase, but dependent on the concentration of acetyl-CoA carboxylase. After the reaction is initiated, the concentration of fatty acid synthetase-bound malonyl-CoA and the velocity of fatty acid synthesis increase from zero to the steady state level in less than a minute when purified enzymes are used.

While performing fatty acid synthetase-coupled assays for acetyl-CoA carboxylase activity in rat liver homogenates, we observed that the reaction

* Address correspondence to: Dr. Richard E. Dugan, Metabolic and Lipid Research Laboratory, William S. Middleton Memorial Veterans Hospital, 2500 Overlook Terrace, Madison, Wisconsin 53705.

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rate gradually increased for more than 3 min, whether we were measuring NADPH oxidation or incorporation of radioactivity from acetyl-CoA into long-chain fatty acids. Evidently a factor was present that exerted a gradually reversible inhibition on the system. We have purified this factor nearly to homogeneity and identified the mechanism of inhibition.

MATERIALS AND METHODS

Chemicals. Pyruvate kinase and lactic dehydrogenase (rabbit muscle), malate dehydrogenase and citrate synthetase (pig heart), NADH, NADPH, phosphoenol pyruvate, β-D(-)fructose, fatty acid–free bovine serum albumin, and L-malic acid were purchased from Sigma; acetyl-CoA, malonyl-CoA, ATP, and Type 3 ADP-hexylagarose from P-L Biochemicals; Sephacryl S-300 and Sepharose 4B from Pharmacia; potassium citrate from Fisher Scientific; DE-23 cellulose from Whatman; Scint A scintillation solution from Packard Instruments; and Aquasol scintillation solution and all radionuclides from New England Nuclear.

Purification of Enzymes. Rat liver acetyl-CoA carboxylase (1) and pigeon liver fatty acid synthetase (2) were purified as previously described. The carboxylase may be proteolytically modified for greater activity by this procedure (3).

Assay of Enzyme Activities. (a) Acetyl-CoA Carboxylase. Three of the four assays used to measure the rat liver enzyme's activity depend on the rate of formation of the reaction product malonyl-CoA, and the fourth on the rate of formation of the other reaction product, ADP. Details of these assays have been reported as indicated in the following: (i) fixation of $[^{14}C]HCO_3^-$ into acetyl-CoA (4); (ii) spectrophotometric assay coupling acetyl-CoA carboxylase–catalyzed malonyl-CoA formation to NADPH oxidation by fatty acid synthetase (5,6) (unless specified otherwise, 1-ml assay mixtures contained 0.32 μg of acetyl-CoA carboxylase and 50 μg of fatty acid synthetase); (iii) radiochemical assay coupling malonyl-CoA formation to the rate of synthesis of ^{14}C-labeled long-chain fatty acids (7); (iv) spectrophotometric assay (ADP formation by acetyl-CoA carboxylase coupled to NADH oxidation by lactic dehydrogenase, via conversion of ADP and phosphoenol pyruvate to ATP and pyruvic acid by pyruvate kinase) (8). (b) Fatty Acid Synthetase. The pigeon liver enzyme was assayed spectrophotometrically by monitoring the rate of NADPH oxidation (2). (c) Malonyl-CoA Decarboxylase. The coupled spectrophotometric assay used has been previously described (9).

Sucrose Density Gradient Centrifugation. Solutions were centrifuged in a swinging bucket Beckman SW41 titanium rotor at 130,000 x g for 19 h at 25°C. Each tube contained potassium phosphate buffer, 200 mM, pH 7.4; EDTA, 1 mM; dithiothreitol, 2 mM, and a gradient of 5–20% sucrose. Before centrifugation, fatty acid synthetase, 2.1 mg, and the transient inhibitor protein, 0.7 mg, were placed on top of gradients, individually and together.

Sephadex G25 Gel Filtration of Mixtures of Transient Inhibitor Protein and Radiolabeled Compounds. Radioactive compounds used were $NaH^{14}CO_3$, 7000 dpm/nmol, in the presence of 4 mM $MgCl_2$ and 4 mM ATP; [1-^{14}C]acetyl-CoA, 111,000 dpm/nmol; $AT^{32}P$, 100,000 dpm/nmol; [2-^{14}C]malonyl-CoA, 104,000 dpm/nmol. Two nanomols of each radioactive compound were mixed with 0.4 mg (ca. 2 nmol) of transient inhibitor protein in 1 ml of Tris-HCl, 50 mM, pH 7.4 containing β-mercaptoethanol, 5 mM. The mixture was filtered through a column of Sephadex G-25 (1.1 x 28 cm) at a flow rate of 0.5 ml/min. Effluent was collected in 1-ml fractions and measured for radioactivity and A_{280}.

Purification of the Protein Producing Transient Inhibition. Rat liver homogenates and soluble cell material were prepared as previously described (4). The transient inhibitor was precipitated by 30% saturated ammonium sul-

fate. (In this and subsequent purification steps, the transient inhibition was monitored by the fatty acid synthetase–coupled spectrophotometric assay.) The precipitate was dissolved and dialyzed in potassium phosphate buffer, 10 mM, pH 7.5, containing EDTA, 1 mM, and β-mercaptoethanol, 5 mM. The inhibitor did not bind on passage through a DEAE-cellulose column (1.5 x 12 cm). Four-milliliter aliquots of the 280 nm–absorbing effluent (40 mg of protein) were filtered through a Sephacryl S-300 column (2.2 x 62 cm) using the same buffer and a flow rate of 40 ml/h. Tubes of effluent containing inhibitor activity were pooled, dialyzed in Tris-HCl, 50 mM, pH 7.4, containing EDTA, 0.5 mM, $MgCl_2$, 2 mM, and β-mercaptoethanol, 5 mM, and chromatographed on an ADP-hexylagarose column (0.5 x 5 cm) at a flow rate of 24 ml/h. After the 280 nm absorbance of the effluent returned to background, the column was eluted with the same buffer supplemented with 0.5 M NaCl. The eluted transient inhibitor was concentrated into the potassium phosphate buffer described above by vacuum dialysis in Schleicher and Schuell collodion bags.

RESULTS AND DISCUSSION

Demonstration of Transient Inhibition of the Fatty Acid Synthetase–Coupled Assay for Acetyl-CoA Carboxylase Activity in Rat Liver Homogenates. Acetyl-CoA carboxylase activity in 250 μg of protein in the 10,000 x *g* supernatant solution of homogenates was assayed radiochemically by coupling to fatty acid synthesis. Assays were terminated at 1-min intervals for 7 min. Minute by minute, the following amounts of palmitic acid were formed: minute 1, 0.5 nmol; 2, 1.1 nmol; 3, 1.5 nmol; 4 and thereafter, 1.7 nmol/min. With purified acetyl-CoA carboxylase, maximum reaction velocity was achieved by the second minute.

Separation of Transient Inhibitor from Copurifying Enzymes. The early steps of purification described above did not separate acetyl-CoA carboxylase and malonyl-CoA decarboxylase from the inhibitor. The carboxylase was removed by DEAE-cellulose and the decarboxylase was partially resolved by Sephacryl S-300 gel filtration and completely separated by ADP-hexylagarose affinity chromatography (Fig. 1). SDS gel electrophoresis of ADP-hexylagarose–purified inhibitor showed a major band of 90,000 and two minor bands of lower molecular weight. The material eluted from Sephacryl S-300 had a molecular weight of approximately 180,000, suggesting that the factor exists as a dimer.

Effect of Transient Inhibitor on Fatty Acid Synthetase–Coupled Spectrophotometric Assay for Acetyl-CoA Carboxylase. The inhibitor's effect on the time course of absorbance at 340 nm is shown in Fig. 2. Constant reaction velocity was attained within 45 sec without inhibitor; with 5 μg of inhibitor, in 2.5 min; and with 50 μg, in 11 min. Like the fatty acid synthetase–coupled radioassay, the coupled spectrophotometric assay yielded a low estimate of acetyl-CoA carboxylase activity in the presence of inhibitor if measured be-

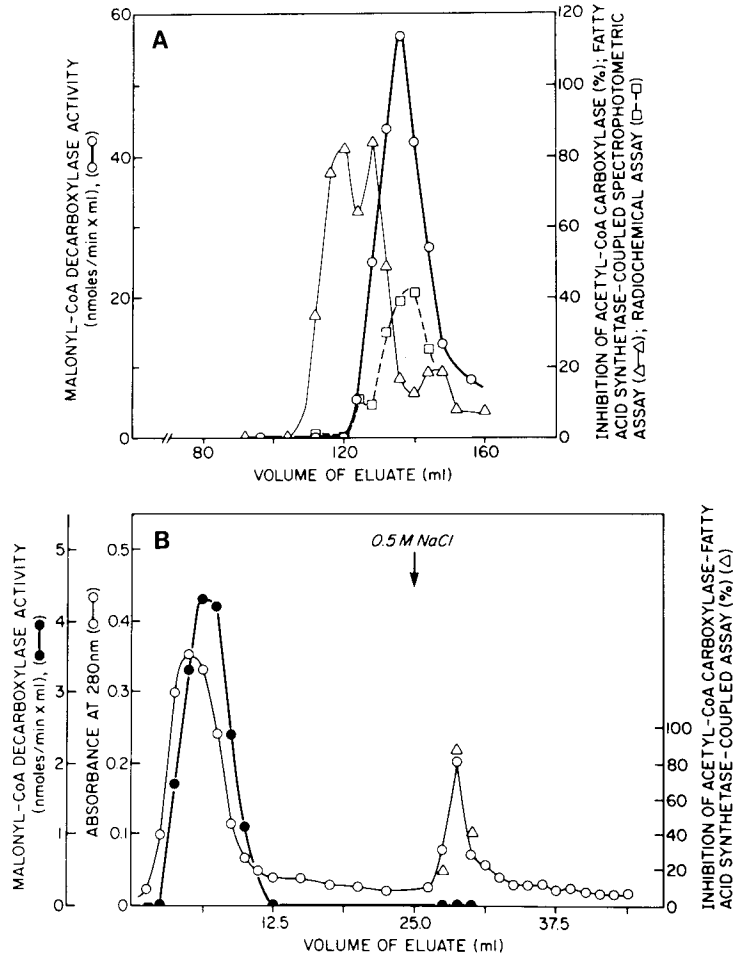

Fig. 1. Gel filtration and affinity chromatography of partially purified homogenates containing malonyl-CoA decarboxylase and the transient inhibitor of fatty acid synthetase-coupled acetyl-CoA carboxylase assays. (A) Sephacryl S-300: 40 mg of protein that did not bind on DEAE-cellulose chromatography was filtered through a gel column (2.2 x 62 cm) at 40 ml/h and collected in 4-ml fractions. Aliquots of 100 µl (bicarbonate fixation assay) and 50 µl (fatty acid synthetase-coupled spectrophotometric assay) were tested for inhibition of acetyl-CoA carboxylase. (B) ADP-hexylagarose: 4 mg of protein that did not bind to DEAE cellulose was chromatographed on an affinity column (0.5 x 5 cm) at 24 ml/h and collected in 1.25-ml fractions. Aliquots of 100 µl (for malonyl-CoA decarboxylase) or 200 µl (inhibition of fatty acid synthetase-coupled spectrophotometric assay for acetyl-CoA carboxylase) were tested.

fore maximum reaction velocity was reached. Furthermore, in the presence of large amounts of the inhibitor, the maximum velocity was reduced (e.g., 50 µg of inhibitor in the presence of 50 µg of fatty acid synthetase reduced the maximum reaction velocity by 50%) (Fig. 2). This may be due to loss of acetyl-CoA carboxylase activity during the extended period of catalysis.

The transient inhibition decreases as the reaction rate increases from

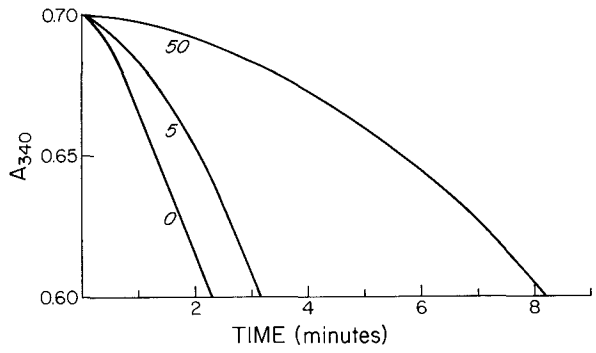

Fig. 2. Absorbance with time in the presence of 0, 5, and 50 µg of the transient inhibitor of the fatty acid synthetase–coupled spectrophotometric assay for acetyl-CoA carboxylase.

zero to a constant value. To express the level of inhibition we have arbitrarily selected the total measured during the first two minutes after initiation of the reaction, when inhibition is most pronounced.

Effect of Transient Inhibitor on Non–Fatty Acid Synthetase–Coupled Assays for Acetyl-CoA Carboxylase Activity. The products of the acetyl-CoA carboxylase–catalyzed reaction are malonyl-CoA and ADP. By coupling ADP formation to NADH oxidation and assaying at different stages of inhibitor purification, we observed that there was no inhibition on this assay for acetyl-CoA carboxylase (Table 1). The apparent inhibitor also had no effect on the bicarbonate fixation assay for malonyl-CoA formation (Table 2). Therefore, the inhibition on

Table 1. Effect of Transient Inhibitor of Fatty Acid Synthetase–Coupled Assays on ADP-NADH–Coupled Assay for Acetyl-CoA Carboxylase

Protein fraction tested for inhibitory activity by ADP-NADH coupled–assay[1]	Acetyl-CoA carboxylase activity (nmol NADH oxidized x min^{-1} x ml^{-1})
None	6.0
Liver soluble protein passed through an avidin Sepharose-linked affinity column	6.1
DEAE-cellulose unbound followed by Sephacryl S-300 gel filtration	5.7
Sephacryl S-300 followed by ADP-hexyl-agarose chromatography (bound)	6.3
DEAE-cellulose bound followed by ADP-hexyl-agarose agarose chromatography (bound)	5.5

[1] The amount of inhibitor from each fraction was that which exerted a 50% inhibition of acetyl-CoA carboxylase activity in the acetyl-CoA carboxylase–fatty acid synthetase–coupled spectrophotometric assay during the first 2 min. Background activity due to ATPases was determined. Then acetyl-CoA was added to samples containing inhibitor, purified acetyl-CoA carboxylase (6 nmol NADH oxidized x min^{-1} x ml^{-1}), and the other assay components.

Table 2. Effect of Transient Inhibitor of Acetyl-CoA Carboxylase–Fatty Acid Synthetase–Coupled Spectrophotometric Assay on Bicarbonate Fixation Assay for Acetyl-CoA Carboxylase Activity

Malonyl-CoA-NADPH–coupled spectrophotometric assay inhibitor[1] (μg)	Enzyme activity	
	Spectrophotometric assay (% of activity in the absence of inhibitor)	$NaHCO_3$ fixation assay (% of activity in the absence of inhibitor)
0	100	100
1.35	88.3	102.7
2.70	67.9	96.5
5.40	54.3	91.4
13.5	32.7	103.9
27.0	21.0	94.1

[1] The spectrophotometric assay inhibitor was purified through ADP-hexylagarose affinity chromatography.

the fatty acid synthetase–coupled assays for acetyl-CoA carboxylase activity is not due to inhibition of acetyl-CoA carboxylase activity.

Effect of Transient Inhibitor on Assay for Fatty Acid Synthesis. The rate of NADPH oxidation by 50 μg of fatty acid synthetase was 56 nmol/min/ml without inhibitor or in the presence of 94 μg of inhibitor. In the coupled assay, this amount of inhibitor reduces the rate of NADPH oxidation more than 90% during the first 2 min. Apparently the factor inhibits an assay coupling two enzymes without inhibiting either enzyme. However, there is a difference in malonyl-CoA concentration in the assays for fatty acid synthesis and fatty acid synthesis coupled to acetyl-CoA carboxylase activity. In the former, malonyl-CoA is 100 μM and saturating; in the latter, the fatty acid synthetase–bound malonyl-CoA concentration is reduced so that NADPH oxidation proceeds at 13% of the velocity when malonyl-CoA is saturating. In the fatty acid synthetase assay at free malonyl-CoA concentrations below 10 μM, fatty acid synthesis appeared to be inhibited by the transient inhibitor of the coupled assay, but quantitation was impossible because without a system for generating malonyl-CoA the reaction rate decreased rapidly as malonyl-CoA was consumed. These results suggest that the transient inhibitor of the coupled assay either competes with fatty acid synthetase for malonyl-CoA or competes with malonyl-CoA for a binding site on fatty acid synthetase.

Test for Comigration of Fatty Acid Synthetase and Malonyl-Co-A With Transient Inhibitor. On sucrose density centrifugation of individual and combined

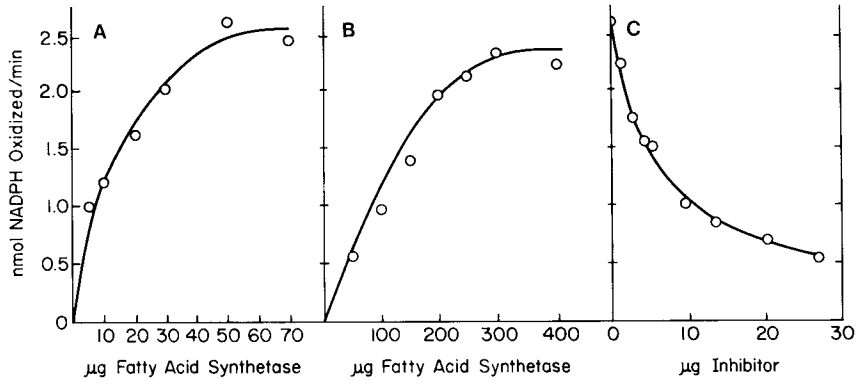

Fig. 3. Effect of combinations of fatty acid synthetase and the transient inhibitor on the fatty acid synthetase–coupled spectrophotometric assay for acetyl-CoA carboxylase. (A) Fatty acid synthetase alone. (B) Fatty acid synthetase in the presence of 27 µg of transient inhibitor. (C) Transient inhibitor in the presence of 50 µg of fatty acid synthetase.

samples of fatty acid synthetase and the transient inhibitor, fatty acid synthetase was located near the bottom of the gradient and the transient inhibitor near the top. The distribution pattern of each protein was unaltered by the presence of the other, indicating no association.

After gel filtration of mixtures of transient inhibitor and selected radiolabeled compounds, radioactivity present with protein indicated that 2 nmol of the transient inhibitor protein had retained 0.50 nmol of malonyl-CoA, 0.09 nmol of ATP, 0.05 nmol of acetyl-CoA, and no bicarbonate. On a second pass through the Sephadex G-25 column the protein retained two-thirds of the malonyl-CoA associated after the first pass. This evidence for affinity for malonyl-CoA supports the idea that the protein could compete with fatty acid synthetase for the binding of malonyl-CoA.

Reversibility of the Inhibition by Fatty Acid Synthetase. The fatty acid synthetase–coupled assay for acetyl-CoA carboxylase was tested as a function of the concentration of fatty acid synthetase without inhibitor and in the presence of 27 µg of inhibitor. In the absence of inhibitor, maximum reaction velocity was reached with 50 µg/ml of fatty acid synthetase (Fig. 3A). However, to reach this reaction velocity in the presence of 27 µg/ml of inhibitor required 300 µg/ml of fatty acid synthetase (Fig. 3B). At low levels of fatty acid synthetase where the reaction rate increases linearly with enzyme concentration, 0.15 µM inhibitor caused a 15-fold reduction in the reaction rate produced by 0.01 µM fatty acid synthetase, indicating a malonyl-CoA binding constant for the inhibitor 12-fold greater than that of fatty acid synthetase.

Figure 3C shows the effect of increasing amounts of inhibitor on the reaction velocity provided by 50 µg/ml of fatty acid synthetase. This pattern is also consistent with fatty acid synthetase and the inhibitor protein competing for the binding of free malonyl-CoA until the inhibitor is saturated with it.

Hypothesized Function of the Protein. The only role for the transient inhibitor consistent with all the data is as a malonyl-CoA-binding protein. This protein might be an enzyme with a regulatory or substrate binding site for malonyl-CoA, or a regulatory protein for enzymes regulated by malonyl-CoA, such as carnitine palmityltransferase (10) and carnitine acyltransferase (11). If it is an enzyme, its affinity for ATP suggests a kinase. Pyruvic kinase is ruled out because the malonyl-CoA-binding protein does not substitute for it in the ADP-coupled assay for acetyl-CoA carboxylase. Biotin-containing enzymes are ruled out because the protein has no affinity for bicarbonate. The protein could serve as a storage depot for malonyl-CoA, but binding data with radioactive malonyl-CoA are more indicative of a single binding site per molecule, which would be inefficient for storage. Hopefully further characterization will identify a physiological function for this protein that inhibits enzyme assays dependent on a low level of free malonyl-CoA. If its in vivo effect is like the in vitro effect reported here, the protein could regulate the tissue malonyl-CoA level, which has been proposed as the coordinator of fatty acid synthesis and oxidation (11,12).

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LITERATURE CITED

1. Inoue, H., and Lowenstein, J.M. (1972) J. Biol. Chem. **247**, 4825-4832.
2. Muesing, R.A., and Porter, J.W. (1975) Meth. Enzymol. **35**, 45-59.
3. Witters, L.A., and Vogt, B. (1981) J. Lipid Res. **22**, 364-369.
4. Abdel-Halim, M.N, and Porter, J.W. (1980) J. Biol. Chem. **255**, 441-444.
5. Numa, S. (1969) Meth. Enzymol. **14**, 9-16.
6. Lornitzo, F.A., Drong, R.F., Katiyar, S.S., and Porter, J.W. (1981) Meth. Enzymol. **71C**, 292-306.
7. Hsu, R.Y., Wasson, G., and Porter, J.W. (1965) J. Biol. Chem. **240**, 3736-3746.
8. Tietz, A., and Ochoa, S. (1962) Meth. Enzymol. **5**, 570-575.
9. Kim, Y.S., and Kolattukudy, P.E. (1978) Arch. Biochem. Biophys. **190**, 585-597.
10. Paulson, D.J., Ward, K.M., and Shug, A.L. (1984) FEBS **176**, 381-384.
11. Robinson, I.N., and Zammit, V.A. (1982) Biochem. J. **206**, 177-179.
12. McGarry, J.D., Takabayashi, Y., and Foster, D.W. (1978) J. Biol. Chem. **253**, 8294-8300.