**LIVER ACETYL COA CARBOXYLASE:
THE DISSOCIATION-REASSOCIATION PROCESS AND ITS RELATION
TO CATALYTIC ACTIVITY***

BY CARLO GREGOLIN,† ELENA RYDER,‡ ROBERT C. WARNER,§
ALBRECHT K. KLEINSCHMIDT, AND M. DANIEL LANE**

DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Communicated by Severo Ochoa, October 3, 1966

In a previous paper¹ we reported that acetyl CoA carboxylase (E.C. 6.4.1.2) isolated in homogeneous form from chicken liver is an unusually large and asymmetric protein structure. The enzyme (in phosphate buffer, pH 7) has a molecular weight of about 8 million and a sedimentation coefficient of 53–58S. Its characteristic filamentous structure, revealed by electron microscopy, was easily dissociated into subunits with concomitant loss of enzymatic activity. Active filaments (70–100 Å × up to 4000 Å) were rapidly reconstituted by addition of isocitrate or other tri- and dicarboxylic acid anions. The present paper provides a more complete physical-chemical characterization of the protomeric and polymeric forms of acetyl CoA carboxylase, as well as an insight into the reversible dissociation-reassociation process and its relation to catalytic activity.

Experimental Procedure.—Acetyl CoA carboxylase was isolated from chicken liver, and carboxylase assays were conducted as described earlier.¹ Except where indicated, protein concentration was determined by the method of Lowry *et al.*² Biotin assays and molecular weight determinations (sedimentation equilibrium) were conducted as previously described.¹

C¹⁴-acetyl CoA-malonyl CoA exchange reaction: C¹⁴-acetyl CoA-malonyl CoA exchange rates were determined in a reaction mixture (0.5 ml total vol) containing the following components (in μ moles, unless specified): Tris (Cl[−]) buffer, pH 7.5, 30; 1-C¹⁴-acetyl CoA (chromatographically purified, sp. act., 4.54×10^5 cpm per μ mole), 0.05; malonyl CoA (chromatographically purified), 0.1; potassium isocitrate, 10; bovine serum albumin, 0.3 mg; EDTA, 0.1; and acetyl CoA carboxylase (sp. act. in the carboxylation assay,¹ 8 units per mg of protein), up to 0.4 μ g. Following a 10-min incubation at 37°, the reaction was terminated and the incorporation of C¹⁴-activity into malonyl CoA determined. Exchange rates were essentially linear for up to 15 min and were proportional to enzyme concentration under the conditions described. The carboxylase-catalyzed exchange reaction exhibits an absolute requirement for isocitrate (or certain other tri- or dicarboxylic acids) under these conditions. Isocitrate, citrate, and malonate are active, whereas tricarballoylate is inactive. The carboxylation rate under comparable conditions is three times greater than the exchange rate.

Amino acid analysis: Amino acid analyses were conducted with a Beckman/Spinco model 120B amino acid analyzer after anaerobic hydrolysis in 5.7 N HCl at 120° for 20 and 40 hr. Destruction of proline, threonine, and serine was corrected for by extrapolation to zero hydrolysis time. Half cystine was determined³ as cysteic acid after performic acid oxidation and hydrolysis; tryptophan was determined spectrophotometrically.⁴

Sucrose density gradient centrifugation: Sucrose density gradients were performed according to the method of Martin and Ames⁵ using a linear 5–20% (w/v) sucrose gradient and a total volume of 5 ml. Additions are indicated in the figure legends. Onto each gradient were applied 30–35 μ g of acetyl CoA carboxylase, specific activity 5–7 units per mg of protein, dissolved in 0.1 ml of a solution having a composition identical to that of the corresponding gradient (minus sucrose). The gradient tubes were centrifuged at 39,000 rpm (SW39 rotor; Spinco model L ultracentrifuge) for 120 min at 19–20°. Eighteen fractions were collected and aliquots assayed for carboxylase activity by the HCl¹⁴O₃[−] fixation method.¹ *E. coli* ribosomes of 30S and 50S were used as external markers.

Results.—*Amino acid, biotin, and refractometric protein analysis of acetyl CoA*

TABLE 1
AMINO ACID COMPOSITION AND BIOTIN CONTENT OF LIVER ACETYL CoA CARBOXYLASE

Amino acid	Residues per subunit molecule*	Amino acid	Residues per subunit molecule*
Lysine	170	Alanine	248
Histidine	94	Half-cystine	44
Arginine	231	Valine	289
Aspartic acid	365	Methionine	112
Threonine	156	Isoleucine	226
Serine	181	Leucine	326
Glutamic acid	426	Tyrosine	124
Proline	181	Phenylalanine	145
Glycine	238	Tryptophan	46

Biotin = 0.93 mole per 409,000 gm protein†

* Subunit mol wt = 409,000.

† Refractometrically determined protein.

carboxylase: Protein concentration was determined by refractometric determination in a synthetic boundary cell using 1.862×10^{-4} as the specific refractive index increment per mg per ml. This figure is the average for several proteins⁶ corrected to 546 m μ and 20°. The relation between $A_{280 \text{ m}\mu}^{1 \text{ cm}}$ and refractometrically determined protein is given by mg protein/ml = $0.86 \times A_{280 \text{ m}\mu}^{1 \text{ cm}}$. This factor is close to that (0.84) calculated from the amino acid composition. Protein concentration determined by the method of Lowry *et al.*² (crystalline bovine serum albumin as standard) can be corrected to refractometrically determined protein concentration by multiplying by 1.24. The partial specific volume of the enzyme calculated⁷ from its amino acid composition is 0.737 cm³ per gm. The amino acid and biotin contents of the purified carboxylase are summarized in Table 1. Since PCMB is a potent inhibitor of the enzyme, it is apparent that the half-cystine content must include some cysteine. The (+) biotin content (1 mole/440,000 gm of refractometrically determined protein) is in good agreement with the subunit molecular weight of 409,000 found for the dissociated enzyme to be described in the next section.

Dissociation of acetyl CoA carboxylase; Characterization of the protomeric unit: Liver acetyl CoA carboxylase in 50 mM phosphate, pH 7 ($s_{20, w} = 54S$ and mol wt, 8.1×10^6) is dissociated into subunits by dialysis against 0.5 M NaCl, 50 mM Tris (Cl⁻), pH 8.0, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol. Sedimentation patterns of the "native" and "dissociated" enzyme are shown in Figure 1. Both patterns exhibit single sedimenting boundaries, which is consistent with dissociation to a single subunit species. The sedimentation coefficient ($s_{20, w} = 13.1S$) of the subunit showed no systematic change over a protein concentration range of 0.5–5.0 mg per ml; however, there is a tendency to aggregate at higher concentrations. The molecular weight of the subunit was determined by sedimentation equilibrium in the same medium employed to dissociate the enzyme for the sedimentation velocity experiments. The subunit molecular weight, based on the results of six experiments at different protein concentrations (0.1–0.5 gm per liter), was found to be $4.09 \pm 0.21 \times 10^5$. Other conditions shown earlier¹ to transform the carboxylase into "small form" in sucrose density gradient centrifugation, failed to dissociate the enzyme at the protein concentrations (2–3 mg per ml) used in the sedimentation velocity experiments. This behavior is attributed to the tendency of the enzyme to aggregate at the higher protein concentration. However, there is good agreement between the $s_{20, w}$ of 13.1S in the analytical ultracentrifuge and sedimentation coefficients of 13–

FIG. 1.—Sedimentation patterns of acetyl CoA carboxylase. (a) Native enzyme in 50 mM K · phosphate, pH 7; protein concentration, 1.54 mg/ml. (b) Dissociated enzyme in 0.5 M NaCl, 50 mM Tris (Cl^-), pH 8.0; protein concentration, 3.2 mg/ml. (c) Reconstituted phosphate enzyme dissociated as in (b) followed by dialysis against and centrifugation in 50 mM K · phosphate, pH 7; protein concentration, 1.05 mg/ml. (d) Reconstituted Tris + isocitrate enzyme dissociated as in (b) followed by dialysis against and centrifugation in 50 mM Tris (Cl^-), pH 7.5, plus 20 mM DL-isocitrate; protein concentration, 0.8 mg/ml. Centrifugation was at 20° at 25,980 rpm in (a), (c), and (d) and 50,740 rpm in (b). Photographs were taken at 8, 24, and 40 min in (a) and 8, 20, and 40 min in (b), (c), and (d). Direction of centrifugation, right to left. All solutions contained 0.1 mM EDTA and 5 mM 2-mercaptoethanol.

TABLE 2
MOLECULAR FORMS OF LIVER ACETYL CoA CARBOXYLASE

Form	$s_{20,w}$ (analytical ultracentrifugation*)	Sedimentation coefficient (sucrose density gradient centrif- ugation)	Molecular weight†
	(S)	(S)	
In 50 mM K·phosphate, pH 7	50-55	50-55	$8.3 \pm 1.1 \times 10^6$
In 50 mM Tris (Cl ⁻), pH 7.5	50-55	13-15	—
In 50 mM Tris (Cl ⁻), pH 7.5 + 20 mM DL-isocitrate	50-55	40-50	$4.1 \pm 0.2 \times 10^6$
In carboxylation assay reaction mix- ture ¹ minus isocitrate	50-55	13-15	—
In carboxylation assay reaction mix- ture ¹ + 20 mM DL-isocitrate	50-55	40-45	—
In 0.5 M NaCl + 50 mM Tris (Cl ⁻), pH 8	13.1	13-15	$4.09 \pm 0.21 \times 10^6$

All solutions contained 0.1 mM EDTA and 5 mM 2-mercaptoethanol; the latter component was omitted when the carboxylation assay mixture was used.

* Protein concentration, 0.5-5 mg per ml; albumin omitted from carboxylation assay reaction mixture.

† Protein concentration, 0.08-0.6 mg per ml.

15S for the "small form" obtained by sucrose density gradient centrifugation in the presence of Tris (Cl⁻), 50 mM, pH 7.5; Tris (Cl⁻), 50 mM, pH 7.5, plus 0.5 M NaCl; or the carboxylation assay reaction mixture minus isocitrate. In these particular experiments avian liver acid fatty synthetase,⁸ *s* (uncorrected) = 12S, was employed as internal marker. This leads us to believe that the "small form" obtained under the conditions described is identical to the 409,000-mol wt subunit. Furthermore, the biotin content of the enzyme, i.e., 1 mole of biotin per 440,000 gm of refractometrically determined protein, is in agreement with the subunit molecular weight.

Reconstitution of acetyl CoA carboxylase from the dissociated form; molecular weights of polymeric forms: Carboxylase subunits, prepared by dissociation of the native enzyme (2-3 mg per ml) in 50 mM Tris (Cl⁻)-0.5 M NaCl, pH 8.0, exhibit absolutely no enzymatic activity when isocitrate is omitted from the assay reaction mixture. In the presence of isocitrate in the assay reaction mixture both the dissociated and "native" carboxylase preparations have equal activities. The sedimentation patterns in Figure 1 ("reconstituted:phosphate" and "reconstituted:isocitrate") document the reconstitution of the dissociated enzyme to "forms" having $s_{20,w}$ values of 54 and 52S. Reconstitution was achieved by overnight dialysis against 50 mM phosphate, pH 7, or 20 mM isocitrate in 50 mM Tris (Cl⁻), pH 7.5, respectively. The reconstituted enzyme preparations had specific activities equal to that of the native enzyme (7-8 units per mg of protein) and exhibited the characteristic "native" filamentous structure¹ in the electron microscope. It should be pointed out that although the two reconstituted forms exhibited nearly identical $s_{20,w}$ values, they appear to have different molecular weights, hence different filament lengths. Table 2, which summarizes the molecular weight data, reveals that the "large forms" in phosphate and in Tris plus isocitrate have molecular weights of approximately 8 and 4 million, respectively. These weights correspond to an average of 20 and 10 subunits (0.4 million mol wt), respectively, in the two polymeric forms. It seems likely that the degree of aggregation, hence filament length (or mol wt) depends on a variety of factors, including the nature of the anion, pH, and ionic strength. The two polymeric forms ("phosphate" and "isocitrate") described constitute two families of differing filament lengths. It is apparent that a complex array of filament lengths is possible.

FIG. 2.—Effect of ATP-Mg on the sedimentation rate of acetyl CoA carboxylase in sucrose density gradients. The complete system (carboxylation assay reaction mixture) contained: 0.06 *M* Tris (Cl^-), pH 7.5; 8 mM MgCl_2 ; 2 mM ATP; 10 mM KHCO_3 ; 0.2 mM acetyl CoA; 20 mM DL-isocitrate; 3 mM GSH; 0.1 mM EDTA; and 0.6 mg/ml bovine serum albumin. Deletions are indicated in the figure.

FIG. 3.—Effect of malonyl CoA on the sedimentation rate of acetyl CoA carboxylase in sucrose density gradients. The complete system contained: 0.06 *M* Tris (Cl^-), pH 7.5; 0.4 mM malonyl CoA; 0.2 mM acetyl CoA; 20 mM DL-isocitrate; 3 mM GSH; 0.1 mM EDTA; and 0.6 mg/ml bovine serum albumin. Malonyl CoA or isocitrate were deleted as indicated in the figure.

Dissociation of acetyl CoA carboxylase by ATP-Mg and malonyl CoA: We earlier observed¹ that the "large form" of carboxylase in phosphate buffer added to the carboxylation assay reaction mixture minus isocitrate is initially active, but decays to zero within 1 min. This decay corresponds to its dissociation into the inactive small form under the influence of the components of the reaction mixture. Addition of isocitrate, but not phosphate, instantly reactivates the enzyme concomitant with reconstitution into the large filamentous form. The factors responsible for the dissociation of the carboxylase under the conditions of the carboxylation assay were investigated in detail by the sucrose density gradient centrifugation technique. It should be recalled¹ that the enzyme sediments as the "small form," in sucrose density gradients containing Tris (Cl^-), pH 7.5, or the components of the carboxylation assay reaction mixture minus isocitrate and albumin. Addition of bovine serum albumin to gradients containing the assay reaction minus isocitrate also leads to the "small form." However, if under the same conditions (albumin present) either Mg^{2+} or ATP is omitted, the carboxylase sediments as the "large form," as illustrated in Figure 2. Omission of acetyl CoA from any of the gradients described has no measurable effect on the sedimentation velocity of the carboxylase. These results indicate that under the assay conditions the combination of ATP and Mg^{2+} , presumably ATP-Mg complex, is responsible for the dissociation of the enzyme into inactive subunits. Isocitrate acts, as evident in Figure 2, by counteracting the dissociating effect of ATP-Mg, thereby maintaining the carboxylase in an active, polymeric state. Phosphate is unable to replace isocitrate in this role. Two possible interpretations of the ATP-Mg-promoted dissociation phenomenon have been considered. In the presence of ATP, Mg^{2+} , and HCO_3^- the enzyme exists in its carboxylated state which may have a greater tendency to dissociate than the

FIG. 4.—Competitive inhibition between malonyl CoA and isocitrate in the carboxylation reaction. Assays were performed as described previously,¹ with the following variations: sp. act. of $\text{KHC}^{14}\text{O}_3$, 5.21×10^6 cpm/ μmole ; isocitrate and malonyl CoA concentrations as indicated in the figure and $0.18 \mu\text{g}$ of carboxylase (sp. act., 4 units/mg of protein). Initial velocities (v) are expressed as $\text{m}\mu\text{moles}$ of HCO_3^- fixed per min.

dation of the mechanism will necessitate further investigation.

Competitive kinetic relationship between malonyl CoA and isocitrate: Since ATP-Mg or malonyl CoA cause dissociation of the carboxylase which is reversed by isocitrate (see preceding section), it was predicted that a competitive kinetic relationship between ATP-Mg or malonyl CoA and isocitrate should be demonstrable. Since both ATP and isocitrate bind Mg^{2+} and since it has not been established whether ATP or ATP-Mg complex is the active species in the carboxylation reaction, the possible competitive relationship between malonyl CoA and isocitrate was investigated initially. Malonyl CoA is a potent inhibitor of both the carboxylation ($K_i = 0.01\text{--}0.04 \text{ mM}$) and C^{14} -acetyl CoA-malonyl CoA exchange ($K_i = 0.04 \text{ mM}$) reactions, as well as a substrate in the latter reaction. Lineweaver-Burk analysis revealed, as shown in Figure 4, that malonyl CoA inhibits the carboxylation reaction competitively with respect to isocitrate. The K_m for isocitrate in the absence of added malonyl CoA is 2.9 mM . It has also been ascertained that inhibition of the carboxylation reaction by malonyl CoA is competitive with respect to acetyl CoA. While the competitive inhibition by malonyl CoA with respect to acetyl CoA is of the "classical" type, the competitive relationship between malonyl CoA and isocitrate can be conveniently interpreted in terms of the allosteric model of Monod *et al.*⁹ This possibility is considered in more detail in the *Discussion*.

Further support of the competitive kinetic relationship between malonyl CoA and isocitrate was obtained by an investigation of the C^{14} -acetyl CoA-malonyl CoA exchange reaction. The ability of isocitrate to overcome inhibition of malonyl CoA is illustrated by the Lineweaver-Burk plot shown in Figure 5. It is interesting that the K_m of 0.59 mM for isocitrate in the exchange reaction at the lowest level of malonyl CoA is one fifth that found in the carboxylation reaction. Inhibition of the exchange reaction by malonyl CoA is also competitive with respect to acetyl CoA (see Fig. 6) as has been shown to be the case with the carboxylation reaction.

Discussion and Summary.—Liver acetyl CoA carboxylase shown earlier¹ to be an

enzyme in its uncarboxylated state. Alternatively, binding of ATP-Mg *per se* by the enzyme may promote the dissociation. The initial interpretation was supported by the observation that malonyl CoA, substituted for ATP, Mg^{2+} , and HCO_3^- as the enzyme carboxylating system in sucrose density gradients, was also capable of dissociating the enzyme (see Fig. 3). However, these results do not exclude the possibility that binding *per se* of either malonyl CoA or ATP-Mg promotes the dissociation of the enzyme. In both cases the dissociation into subunits is prevented by isocitrate. While it is clear that ATP-Mg or malonyl CoA promote the dissociation of acetyl CoA carboxylase into protomeric units, the eluci-

Fig. 5.—Competitive inhibition between malonyl CoA and isocitrate in the exchange reaction. C^{14} -acetyl CoA-malonyl CoA exchange rates have been measured as described in *Experimental Procedure* with the following variations: DL-isocitrate and malonyl CoA concentrations as indicated in the figure and $0.52 \mu\text{g}$ of carboxylase (sp. act., 5 units/mg of protein). Initial velocities (v) are equivalent to μmoles of C^{14} -acetyl CoA incorporated into malonyl CoA per min.

Fig. 6.—Competitive inhibition between malonyl CoA and acetyl CoA in the exchange reaction. C^{14} -acetyl CoA-malonyl CoA exchange rates have been measured as described in *Experimental Procedure* with the following variations: concentrations of acetyl CoA are indicated on the abscissa (each tube contained 1- C^{14} -acetyl CoA equivalent to 22,700 cpm); 0.1, 0.2, and 0.4 mM malonyl CoA and $0.26 \mu\text{g}$ of carboxylase (sp. act., 5 units/mg of protein). Initial velocities (v) are equivalent to μmoles of C^{14} -acetyl CoA incorporated into malonyl CoA per min.

exceptionally large and filamentous structure in the presence of phosphate or isocitrate has been dissociated, with $0.5 M$ NaCl- 50 mM Tris, pH 8, into inactive protomeric units. The molecular weight of the subunit by sedimentation equilibrium is 409,000 and its sedimentation coefficient ($s_{20, w}$) is $13.1S$. Since the forms of the carboxylase in phosphate and isocitrate have average molecular weights of 8.3 and 4.1 million, respectively, it is apparent that they are composed, on the average, of twenty and ten 409,000-mol wt subunits, respectively. The biotin content of the enzyme corresponds to 1 mole of biotin per 440,000 gm of refractometrically determined protein and is in a 0.93:1 ratio with the subunit molecular weight. The subunit form has been reconstituted to the catalytically active polymeric forms by equilibration with phosphate, pH 7, or Tris plus isocitrate, pH 7.5. As reported earlier,¹ in the presence of the carboxylation assay reaction mixture without isocitrate the enzyme sediments in density gradients as a "small" structure ($13\text{--}15S$). Evidence is presented in this paper which indicates that this form is identical to the 409,000-mol wt subunit. The factor responsible for the dissociation of the carboxylase in the presence of the components of the assay reaction mixture (minus isocitrate) has been identified as ATP-Mg. Since malonyl CoA has also been found to promote the dissociation of the enzyme, it is conceivable that "carboxylated" enzyme has a greater tendency to dissociate than the "uncarboxylated" enzyme. When isocitrate is added, a condition which gives rise to the active form of the carboxylase, the enzyme sediments as a large structure of about $40S$ either in the presence of the carboxylation assay reaction mixture or malonyl CoA. Consistent with these findings is the observation that there is a competitive kinetic relationship between malonyl CoA and isocitrate.

While carboxylation of the enzyme may promote its dissociation, other factors, such as alkaline pH or the presence of certain salts, are also effective in this regard. In these cases isocitrate favors the reconstitution of the active polymeric form. The results presented in this and a previous paper¹ are interpretable in terms of the following schematic model (cf. Monod *et al.*⁹):

In this model we propose the existence of a "modified small form" in equilibrium with the "small form" and whose characteristic conformation permits linear aggregation giving rise to the "large form." Although it is established that the "large form" is catalytically active while the "small form" is not, no definite statement can be made regarding the activity of the hypothetical "modified small form." Factors known to promote the formation of the "large form" act either by shifting the equilibrium through preferential binding to the "modified small form" or to the "large form," or by altering the equilibrium state.

The authors wish to express their appreciation to Dr. Severo Ochoa for his encouragement during the course of this investigation, and to Miss Inge Grumm for her skillful technical assistance. The assistance of Mr. Frank Zaboretsky with the ultracentrifuge experiments is gratefully acknowledged.

* This investigation was supported by research grants AM-09116 and GM-06967 from the National Institutes of Health, U.S. Public Health Service.

† Recipient of a NATO Fellowship (1965), on leave of absence from the University of Padua, Padua, Italy.

‡ Postdoctoral fellow of Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela.

§ Career Development Awardee (U.S. Public Health Service Research Career Program Award, 2-K3-14899).

** Career Development Awardee (U.S. Public Health Service Research Career Program Award, K3-AM-18487).

¹ Gregolin, C., E. Ryder, A. K. Kleinschmidt, R. C. Warner, and M. D. Lane, these PROCEEDINGS, **56**, 148 (1966).

² Lowry, N. O., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

³ Moore, S., *J. Biol. Chem.*, **238**, 235 (1963).

⁴ Goodwin, T. W., and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

⁵ Martin, R. G., and B. N. Ames, *J. Biol. Chem.*, **236**, 1372 (1961).

⁶ Perlman, G. E., and L. Longworth, *J. Am. Chem. Soc.*, **70**, 2719 (1948).

⁷ Cohn, E. J., and J. T. Edsall, *Proteins, Amino Acids, and Peptides* (New York: Reinhold Publishing Corp., 1943), p. 370.

⁸ Hsu, R. Y., G. Wasson, and J. W. Porter, *J. Biol. Chem.*, **240**, 3736 (1965).

⁹ Monod, J., J. Wyman, and J. P. Changeux, *J. Mol. Biol.*, **12**, 88 (1965).