A new method for the isolation of rat liver acetyl-CoA carboxylase

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Summary Rat liver acetyl-CoA carboxylase has been purified to homogeneity by a new method involving polyethylene glycol precipitation, and DEAE and Sepharose 4B chromatography. The final product displays a single band on SDS polyacrylamide gel electrophoresis of estimated molecular weight 240,000. This material contains 5.5 ± 0.3 moles of alkali-labile phosphate per subunit and has a specific activity of 1.2 ± 0.2 units per mg protein. As compared to previous purification procedures for the liver enzyme, this product has a higher phosphate content, lower specific activity, and an absence of major proteolysis. Trypsin digestion of ^{32}P-labeled acetyl-CoA carboxylase from hepatocytes reveals that the ^{32}P-labeled phosphorylation sites are extremely labile to proteolytic digestion. Potential modification of isolated liver acetyl-CoA carboxylase by proteolysis and/or dephosphorylation must be ascertained prior to in vitro enzymatic studies.—**Witters, L. A., and B. Vogt.** A new method for the isolation of rat liver acetyl-CoA carboxylase. *J. Lipid Res.* 1981. **22:** 364–369.

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Acetyl-CoA carboxylase (E.C. 6.4.1.2) is generally regarded as a key rate-limiting enzyme of fatty acid biosynthesis. Acute regulation of enzymatic activity appears to be accomplished both by allosteric effectors (1) and covalent phosphorylation (2–7). In order to fully characterize the enzyme with respect to its kinetic properties and to delineate further its important protein kinases and protein phosphatases, highly purified enzyme is necessary. One of the major handicaps to such characterizations has been major proteolysis occurring during isolation of the enzyme from either chicken or rat liver. Tanabe, et al. (8) and Mackall and Lane (9) have previously provided convincing evidence of this proteolysis. They and other investigators (10) have reported that isolated acetyl-CoA carboxylase of high specific activity may display up to three bands on SDS polyacrylamide gels. The major band has a reported molecular weight of 215,000–230,000 daltons, but other bands of 118,000 and 125,000 are also evident. While early studies concluded that the smaller molecular weight bands were distinct subunits, more recent investigators (8, 9) strongly suggest that these bands arise from proteolysis of the major 215,000–230,000 molecular weight band. Indeed, the enzyme isolated from the rabbit mammary gland contains only a single subunit of molecular weight 252,000 (11). A further complication of proteolysis modification has been the demonstration that trypsin treatment of purified enzyme leads to enzyme activation (12).

A second issue with respect to the isolation of acetyl-CoA carboxylase is preservation of covalently bound phosphate. This concern is especially applicable to the interpretation of studies of in vitro phosphorylation or dephosphorylation. There is only a single report of the phosphate content of the liver enzyme. Inoue and Lowenstein (10) reported that the rat liver enzyme contained 2.1 moles of phosphate per mole of subunit. Recent studies in the rabbit mammary gland (4) indicate that the omission of fluoride during the enzyme isolation procedure leads to dephosphorylation and activation of the enzyme during the isolation. The phosphate content falls from 6.2 ± 0.2 moles P_i/mole subunit (presence of F^-) to 4.8 ± 0.3 (absence of F^-), associated with an increased specific activity of the final product from 1.2 to 3.6 units/mg protein. Thus, given the currently published isolation technique for the liver enzyme, it seems quite likely that both major proteolysis and dephosphorylation occur during the isolation procedure, rendering a final product which may be unsuitable for in vitro studies.

In this study, we report the development of a new isolation procedure for rat liver acetyl-CoA carboxylase. This procedure done in the presence of F^- and entirely at 4°C minimizes major proteolysis and is associated with a greater preservation of phosphate content. In addition, we report the isolation of ^{32}P-labeled acetyl-CoA carboxylase from ^{32}P-labeled rat hepatocytes and provide a preliminary characterization of the lability of the ^{32}P-labeled phosphorylation sites.

MATERIALS AND METHODS

Enzyme isolation

Male Sprague-Dawley rats weighing between 175–225 g were fasted for 72 hr and refed for 48 hr with a low fat, high carbohydrate diet (Fat-free Test Diet, ICN Pharmaceuticals). Ten to twenty rats were killed

Abbreviations: PEG, polyethylene glycol; ACC, acetyl-CoA carboxylase.

by cervical dislocation in groups of five or six. All procedures were carried out at 4°C. The livers were quickly removed and rinsed in homogenization buffer (potassium phosphate 50 mM (pH 7.50) containing 50 mM sodium fluoride, 2 mM EDTA, 10 mM β-mercaptoethanol, and 0.25 M sucrose). The livers were then homogenized in the same buffer (1:2 w/v) with a Waring blender at top speed for 1 min. The homogenate volume was measured and brought to 3% polyethylene glycol (PEG) (w/v) by the slow addition of a 50% PEG-6000 solution while gently stirring. The mixture was allowed to sit for 20 min on ice and was then centrifuged at 20,000 *g* for 20 min. The supernatant was removed and filtered through glass wool. The 3% PEG supernatant was then raised to 5% PEG with an additional amount of 50% PEG while gently stirring. This remained on ice for 20 min and was then centrifuged at 20,000 *g* for 20 min. The supernatant was discarded, and the pellet resuspended in buffer (about 1/8 of starting volume) containing 90 mM potassium phosphate, pH 7.5, 50 mM sodium fluoride, 2 mM EDTA, 10 mM sodium citrate, and 10 mM β-mercaptoethanol, using an Ultraturrax homogenizer at low speed. Following resuspension, the mixture was centrifuged at 20,000 *g* for 15 min to remove insoluble material. The supernatant was filtered through glass wool.

The filtrate was then applied to a DEAE column (1.2 × 45 cm) equilibrated with the same buffer. The column was washed with 200 ml of buffer after sample application and then a linear phosphate gradient elution was carried out with 200 ml of starting buffer and 200 ml of potassium phosphate 500 mM buffer of otherwise identical composition. The elution of acetyl-CoA carboxylase was determined by assay of the gradient fractions (see below); in general, all of the acetyl-CoA carboxylase was eluted at a conductivity of <20 mmho. After assay, all fractions containing acetyl-CoA carboxylase were pooled, the volume was measured, and the solution brought to 50% saturation with NH_4SO_4 (at 4°C) by the slow addition of solid NH_4SO_4. The pH was kept constant at 7.4 with 5 N KOH during the NH_4SO_4 addition. The suspension was allowed to sit on ice for 30 min, and the pellet was collected by centrifugation at 20,000 *g* for 20 min. The NH_4SO_4 pellet was resuspended in 4–5 ml of buffer used for the Sepharose column (see below), and dialyzed for 2 hr against 6 liters of the same buffer at 4°C. Following dialysis, the material was spun at 20,000 *g* for 10 min to remove insoluble material.

This material was then applied to a Sepharose 4B column (2.5 × 90 cm) equilibrated with a buffer containing potassium phosphate, 50 mM, sodium fluoride, 50 mM, EDTA, 2 mM, sodium citrate, 10 mM, and β-mercaptoethanol, 10 mM, and eluted at a flow rate of approximately 0.6 ml/min; fractions of 4–5 ml were collected. Acetyl-CoA carboxylase is included in the column and elutes with an apparent molecular weight of ~5–10 × 10^6 daltons. All active fractions (by assay) were pooled, the total volume was measured, and the material was again brought to 50% saturation with solid NH_4SO_4. The NH_4SO_4 pellet was resuspended in the Sepharose buffer (2–3 ml) and dialyzed overnight at 4°C against the same buffer containing 20% glycerol (v/v).

Isolated acetyl-CoA carboxylase was stored at −20°C after dialysis. The purified enzyme appears to be stable for at least 2 months under these conditions without evidence of proteolysis or loss of activity. Omission of the glycerol was associated with a rapid loss of activity and precipitation of the material.

Acetyl-CoA carboxylase was assayed by a previously published method (13). Assays were performed at 5 mM Mg^{2+} and 5 mM citrate, which are the maximally activating concentrations under the conditions of this assay. Activity is expressed as units per mg protein, where 1 unit = 1 μmole $H^{14}CO_3^-$ fixed to malonyl CoA per minute.

Protein was determined by the method of Lowry, et al. (14). Alkali-labile phosphate was determined by the method of Nimmo and Cohen (15), as modified by Dr. Graham Hardie and co-workers.[1]

^{32}P-Labeled acetyl-CoA carboxylase was isolated from ^{32}P-labeled hepatocytes. Hepatocytes were prepared and incubated with $^{32}P_i$ by methods described previously (16). Preliminary experiments indicated that a 1-hr incubation of hepatocytes with $^{32}P_i$ was associated with steady-state labeling of ^{32}P-labeled acetyl-CoA carboxylase, as judged by scanning densitometry of radioautographs from dried polyacrylamide gels of isolated 100,000 *g* cell supernatant.

SDS-polyacrylamide gel electrophoresis was carried out in slab and cylindrical gels by the method of Neville (17) and Laemmli (18). For analysis of the isolated enzyme, 7.2% (pH 9.81) and 5.6% (pH 9.18) Neville gels were employed. For analysis of patterns of tryptic digestion, 15% Laemmli slab gels were used. The latter were stained and destained as described previously (16), dried with the use of a Bio-Rad gel dryer and radioautographed at −70°C with an intensifying screen and Kodak XR-5 film.

Trypsin digestion of the native ^{32}P-labeled enzyme was carried out at 37°C by the addition of 1% trypsin (by weight) to an aliquot of isolated enzyme

[1] Personal communication.

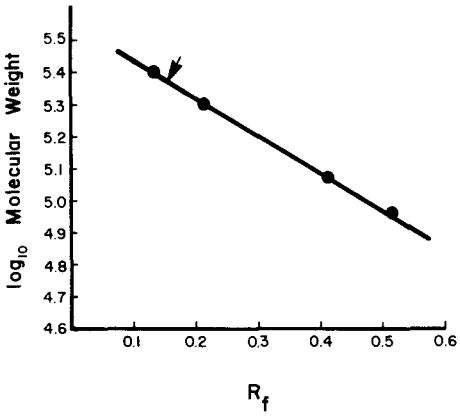

Fig. 2. Molecular weight standard curves. The molecular weight of isolated rat liver acetyl-CoA carboxylase was determined employing 5.6% Neville gels (pH 9.18). The arrow indicates the position of the rat liver enzyme run in the presence of standards. The molecular weight standards employed were rabbit mammary acetyl-CoA carboxylase (252,000; see reference 11), murine cardiac myosin (200,000), β-galactosidase (118,000), and glycogen phosphorylase (94,000).

the predominant 240,000 species with only traces of the 118,000 and 125,000 proteolytic fragments.

Other workers have emphasized the problems of enzyme proteolysis occurring during the isolation of the liver enzyme. These studies demonstrate a large molecular weight subunit of 215–230,000 daltons, but also two other components of molecular weight 118,000 and 125,000 (**Table 2**). Tanabe, et al. (8) and Mackall and Lane (9) have provided clear evidence that the two lower molecular weight bands result from proteolytic cleavage of the larger subunit. Employing our purification procedures, we have generally minimized or avoided generation of these proteolytic fragments during isolation. The reasons for the avoidance of major proteolysis are not entirely clear. All procedures are performed at 4°C, while other procedures (10, 19, 20) are done at room temperature. The use of $CaPO_4$ in the isolation may lead to the activation of cation-dependent proteases (11). We have also observed the isolation of the enzyme beginning with 3% PEG precipitation of a 100,000 *g* supernatant usually leads to the appearance of the small molecular weight fragments in the final product. Thus, the rapid initial 3% PEG precipitation of the liver homogenate may be contributing to the avoidance of proteolysis that may be occurring during an initial high-speed centrifugation.

Because our final product is slightly smaller than the rabbit mammary enzyme and because Hardie and Cohen (11) have suggested that minor proteolysis yielding fragments of 230,000 and 240,000 daltons may occur during isolation of the mammary enzyme, we cannot discount the possibility that the rat liver enzyme has indeed undergone minor proteolysis during purification. The inclusion of several proteolytic inhibitors (phenylmethylsulfonyl fluoride, leupeptin, pepstatin, benzamidine, *O*-phenanthroline) either alone or in combination through the entire isolation procedure does not alter the size of the final product we obtain (not shown). Hardie and Guy[1] have recently noted that the rat mammary enzyme is also 240,000 daltons; this suggests that there may be a true species difference between rabbit and rat in the molecular weight of the enzyme.

Our final product contains 5.5 ± 0.3 (SD) moles of alkali-labile phosphate per mole of 240,000 subunit. This phosphate content is considerably

TABLE 2. Comparison of methods of purification of acetyl-CoA carboxylase

Investigator	Ref.	Source	Specific Activity[a] (U/mg)	"Subunit" Size	Phosphate Content (moles P_i/mole subunit)[b]
Gregolin and Lane	19	Chicken liver	11.0	110–120,000	NR[c]
Inoue and Lowenstein	10	Rat liver	14.4	215,000 125,000 118,000	2.1
Nakanishi and Numa	20	Rat liver	15.1	230,000 124,000 118,000	NR
Hardie and Cohen	4	Rabbit mammary gland	1.2	252,000	6.2 ± 0.2
Witters and Vogt	Present report	Rat liver	1.2	240,000	5.5 ± 0.3

[a] Enzyme specific activity has been corrected to the $H^{14}CO_3^-$ fixation assay at 37°C.
[b] Phosphate content is expressed as moles inorganic phosphate per mole large subunit (215–252,000).
[c] NR indicates not reported.

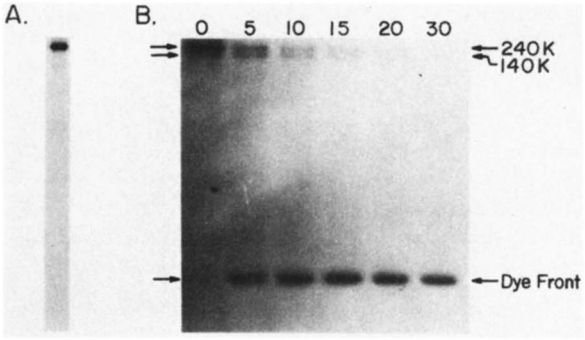

Fig. 3. Isolation and tryptic digestion of ^{32}P-labeled acetyl-CoA carboxylase displayed in a composite radioautograph. Panel A depicts the homogeneity of ^{32}P-labeled acetyl-CoA carboxylase purified from ^{32}P-labeled hepatocytes and analyzed on 7.2% Neville gels at pH 9.81. Panel B depicts a time course of trypsin digestion (0–30 min) of ^{32}P-labeled acetyl-CoA carboxylase. Digestion was carried out as described in Methods. The products were analyzed on a 15% Laemmli slab gel.

higher than previously reported for the rat liver enzyme but compares favorably to that reported for the rabbit mammary enzyme (Table 2). The loss of covalently bound structural phosphate that may occur during enzyme isolation is likely to be due to either dephosphorylation by protein phosphatases and/or proteolysis. Hardie and Cohen (4) have demonstrated that rabbit mammary enzyme isolated in the absence of F^- contains ~2 moles P_i/mole subunit *less* than the enzyme isolated in the presence of F^-. Of additional interest was the observation that this phosphate loss was associated with apparent activation of the enzyme during isolation, yielding a final product of apparent higher specific activity. Omission of NaF from our isolation procedure also is associated with a higher specific activity of the final product (4.0 ± 0.5 U/mg protein).

Proteolysis may also lead to loss of structural phosphate during isolation. The alkali-labile phosphate content of our final product prepared either in the absence *or* presence of the above proteolytic inhibitors was identical. However, in order to gain insight into the potential proteolytic lability of this phosphate, the enzyme has been isolated from hepatocytes after incubation with $^{32}P_i$. ^{32}P-Labeled enzyme was isolated by a procedure identical to that described for the unlabeled whole liver enzyme. A radioautograph of the final product is shown in **Fig. 3A.** Preliminary data based on measurement of cellular ATP^{32} specificity activity and the ^{32}P content of the final product indicate that 3–4 moles of ^{32}P are incorporated into 1 mole of 240,000 subunit following a one-hour incubation of hepatocytes with $^{32}P_i$.[2] This compares favorably with the number of moles of alkali-labile phosphate per mole protein and would suggest that the bulk of the structural phosphate is turning over rapidly.

Isolated ^{32}P-labeled acetyl-CoA carboxylase has been subjected to trypsin digestion and the products were analyzed by 15% polyacrylamide gels. Fig. 3,B shows a time course of such a trypsin digestion. The labeled enzyme used in this experiment, while apparently homogenous by Coomassie staining on gel analysis, does show some proteolysis with appearance of a ^{32}P-labeled band of ~140,000 daltons. This smaller molecular weight band, along with the 240,000 dalton band, are both specifically immunoprecipitated by acetyl-CoA carboxylase anti-serum (not shown), Thus, the substrate is likely partially proteolyzed. Nonetheless, the experiment demonstrates the rapid loss of ^{32}P from the 240,000 dalton subunit with appearance of all the ^{32}P in peptides migrating near the gel dye front (molecular weight ≤ 6000). Other experiments (not shown) indicate that this ^{32}P loss occurs within 1–2 min of exposure of the native enzyme to 1% trypsin and that there is no evidence by gel analysis of intermediate-sized ^{32}P-peptides generated during the course of trypsin digestion. These data suggest that the ^{32}P-labeled sites probably reside close to the amino or carboxyl terminus of the 240,000 dalton peptide.

The specific activity of ACC isolated by the present procedure (1.2 ± 0.2 U/mg protein) is considerably lower than that previously reported for isolated rat (10, 20) or chicken liver enzyme (19). However, it does compare favorably with that of the rabbit mammary gland (4) (Table 2). Because of dephosphorylation of ACC leads to activation of the enzyme and an apparent increase in specific activity, it seems likely that previous procedures for the isolation of the liver enzyme have been accompanied by dephosphorylation, either through the action of protein phosphatases or through limited proteolysis. Thus, the term "high specific activity" seems inappropriate when applied to enzymes that can be interconverted via phosphorylation/dephosphorylation, because phosphate loss during the isolation of such enzymes could lead to higher specific activity of the final product, which has been substantially modified. Homogeneity demonstrated by SDS polyacrylamide gel analysis coupled with determination of covalently bound phosphate, in our view, are necessary to information with regard to the characterization of isolated interconvertible enzymes of this nature.

In summary, we report a new method for the isolation of rat liver ACC which results in a homogenous product free from major proteolysis with a higher

[2] Kowaloff, E. M., and L. A. Witters. Unpublished observations.

phosphate content than previously reported. However, because of the evident lability of the phosphate to proteolysis and the size of the final product as compared to the rabbit mammary enzyme, we continue to be concerned about possible loss of potential phosphorylation sites during isolation. Studies of the in vitro phosphorylation or dephosphorylation of liver ACC must take into account potential modification of the enzyme substrate during isolation.

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