Acetyl-Coenzyme A Carboxylase

ROLE OF THE PROSTHETIC GROUP IN ENZYME POLYMERIZATION

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Apo-(acetyl-CoA carboxylase) completely free from the holoenzyme was prepared from biotin-deficient rat adipose tissue by using affinity chromatography. The apoenzyme does not aggregate under conditions favouring the transition of the holoenzyme to the polymeric form. Such transition is possible after the conversion of the apoenzyme into the holoenzyme *in vitro*, thus demonstrating the requirement of the prosthetic biotinyl group for enzyme activation.

The catalytic activity of acetyl-CoA carboxylase [acetyl-CoA-CO₂ ligase (ADP forming), EC 6.4.1.2] appears to be intimately associated with its state of aggregation in that the enzyme is capable of oscillating between the polymeric form of high activity and the protomeric form of low activity. Vagelos and co-workers (Martin & Vagelos, 1962; Vagelos *et al.*, 1963) were the first to show that the stimulatory effect of citrate on fatty acid synthesis is through activation of acetyl-CoA carboxylase. This was shown to be associated with an increase in the sedimentation velocity of the enzyme (Vagelos *et al.*, 1963; Matsushashi *et al.*, 1964; Numa *et al.*, 1965). The conditions favouring respectively the aggregation and disaggregation of acetyl-CoA carboxylase have been listed by Gregolin *et al.* (1972). In view of the correlation between citrate-mediated catalytic activity and citrate-mediated molecular aggregation Lane and coworkers (Gregolin *et al.*, 1972; Moss & Lane, 1972) have suggested that changes in cellular citrate concentrations control the rate of the carboxylase reaction. We present evidence to show that the biotinyl prosthetic group of holo-(acetyl-CoA carboxylase) is essential for the transition of the protomeric to the polymeric form of the enzyme.

Experimental

Materials

CNBr-activated Sepharose, Sephadex G-10, Sephadex G-25 and Sepharose 4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Avidin and Carbowax respectively were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and Union Carbide Corp., Chicago, Ill., U.S.A. D(+)-[carbonyl-¹⁴C]Biotin and NaH¹⁴CO₃ were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. All other chemicals were ob-

tained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Separation of protomeric and polymeric enzyme on a column of Sepharose 4B

Weanling male rats were made biotin-deficient by feeding them an avidin diet (Dakshinamurti & Desjardins, 1968) for 4 to 6 weeks. The epididymal adipose tissue (8.4g) was homogenized in 3 vol. of buffer A (0.1M-Tris, pH 7.5; 0.15M-KCl; 0.1mM-EDTA; 5mM-2-mercaptoethanol) and the high-speed supernatant (105000g, 60min) was prepared. This was passed through a column (2.5cm×45cm) of Sephadex G-25. The eluate was brought to 35% satn. with (NH₄)₂SO₄ and after being left for 30min the suspension was centrifuged for 45min at 13000g. The precipitated protein was divided into two portions, one of which was dissolved in buffer B (0.1M-Tris, pH 8.2; 0.15M-KCl; 0.1mM-EDTA; 5mM-2-mercaptoethanol) and the other in buffer C (0.1M-potassium phosphate, pH 6.9; 0.1mM-EDTA; 5mM-2-mercaptoethanol; 10mM-citrate). The protein dissolved in buffer C was incubated at 37°C for 1h. After this the enzyme preparations were passed through a column (2.5cm×90cm) of Sepharose 4B, by using the same buffer in which the protein was dissolved, for elution. Fractions of volume 3ml were collected and acetyl-CoA carboxylase activity was determined in portions under conditions favouring aggregation (Desjardins & Dakshinamurti, 1971a).

The enzyme fraction was preincubated for 30min at 37°C in a medium containing the following reagents, in a final volume of 1ml: 60mM-Tris, pH 7.5; 5mM-2-mercaptoethanol; 0.1mM-EDTA; 5mM-potassium citrate and 0.6mg of bovine serum albumin. After preincubation carboxylase activity was determined in samples of volume 0.2ml by a further incubation for 10min at 37°C in a medium containing the following reagents, in a final volume of 0.25ml:

60mM-Tris, pH 7.5; 5mM-2-mercaptoethanol; 0.1 mM-EDTA; 5mM-potassium citrate; 0.6mg of bovine serum albumin/ml; 2mM-ATP; 0.2mM-acetyl-CoA; 10mM- $\text{NaH}^{14}\text{CO}_3$ (1 μCi). The reaction was terminated by addition of 0.05ml of 6M-HCl. A sample (0.2ml) was transferred to a scintillation vial, evaporated to dryness and the residue suspended in 0.2ml of water. To this 15ml of a toluene-ethanol scintillator (Dakshinamurti & Desjardins, 1968) was added and radioactivity was determined in a Beckman model LS-250 liquid-scintillation spectrometer.

In the experiment where the enzyme was separated on the column under conditions favouring aggregation, fractions marked X and Y (Fig. 1) were pooled. The holo- and apo-enzyme activities of the pooled fractions were determined respectively both in the presence and in the absence of a holo-(acetyl-CoA carboxylase) synthetase, a preparation essentially free from carboxylase activity (Desjardins & Dakshinamurti, 1971a). A sample of the enzyme preparation was incubated for 4h at 30°C in a medium containing the following reagents in a volume of 0.95ml: 3.6mM- MgCl_2 ; 9.9mM-potassium citrate; 0.72mg of bovine serum albumin/ml; 4mM-ATP; 82 μM -biotin; 1mg of holoenzyme synthetase. At the end of this acetyl-CoA and $\text{NaH}^{14}\text{CO}_3$ (1 μCi) were added in final concentrations of 0.2mM and 10mM respectively, and then incubated further for 10min. The reaction was terminated by 0.05ml of 6M-HCl and the acid-stable radioactivity was determined as described above.

Conversion of apo-(acetyl-CoA carboxylase) into the holoenzyme in vitro

In a further experiment [^{14}C]biotin was incorporated into apo-(acetyl-CoA carboxylase) and the protomer-polymer transition of the labelled enzyme monitored by using the calibrated Sepharose 4B column. The apoenzyme was prepared by affinity chromatography on a Sepharose-avidin column described previously (Landman & Dakshinamurti, 1973). In this procedure, briefly, the 0-35%-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate, prepared as described above, was dissolved in 3ml of buffer C to give a protein concentration of 28mg/ml. This was passed through the Sepharose-avidin column (0.8cm $\times$ 2.0cm) and eluted with buffer C. The eluate (12ml) was concentrated by the use of Carbowax to 4ml to give a 17mg/ml protein concentration of the apoenzyme preparation.

Sepharose 4B chromatography of the apoenzyme preparation. The apoenzyme preparation was applied on the pre-calibrated column (2.5cm $\times$ 85cm) of Sepharose 4B, eluted with buffer C and fractions of volume 3ml were collected. They were assayed for acetyl-CoA carboxylase in presence of biotin and holoenzyme synthetase. Fractions 21-25 (corresponding to Y of Fig. 1) were pooled and concentrated by using Carbowax to a protein concentration of 3.1 mg/ml.

Synthesis of acetyl-CoA carboxylase holoenzyme in vitro

The apoenzyme preparation was incubated for 4h at 30°C in the following medium: 60mM-Tris, pH 7.5; 4mM-ATP; 8mM- MgCl_2 ; 81 μM -D(+)-[carbon- ^{14}C]biotin (18.1×10^6 d.p.m./88nmol); 12.4 mg of apoenzyme and 1.6mg of holoenzyme synthetase. At the end of this, the incubation mixture was dialysed against 4 litres of buffer B (favouring the protomeric form of the enzyme) for 8h. It was then passed through a column (0.9cm $\times$ 50cm) of Sephadex G-10 and eluted with buffer B. The eluate was brought to 50% satn. with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was dissolved in 1ml of buffer B. It was applied on the calibrated column of Sepharose 4B and eluted with buffer B. The fractions (3ml) were monitored for radioactivity, which was recovered entirely in fractions 28-32. To the pooled fraction 12.4 mg of protomeric acetyl-CoA carboxylase was added and dialysed at room temperature for 8h against 4 litres of buffer C (favouring aggregation). The dialysate was concentrated to 1ml by using Carbowax and applied on the column of Sepharose 4B and eluted with buffer C. Fractions (3ml) were collected and radioactivity was monitored.

Protein was determined as described by Lowry *et al.* (1951).

Results

Molecular sieving using chromatography on a column of Sepharose 4B was used to separate the protomeric (mol.wt. 4×10^5) form of holo-(acetyl-CoA carboxylase) from the polymeric form of the enzyme

Fig. 1. Separation of protomeric and polymeric enzyme on a column of Sepharose 4B

A column (2.5cm $\times$ 85cm) of Sepharose 4B was used and fractions (3ml) were collected. All fractions were assayed for acetyl-CoA carboxylase as described in the text. ●, Enzyme preparation under protomeric conditions was eluted with buffer B; ■, enzyme preparation under polymeric conditions was eluted with buffer C.

Table 1. Separation of apo- and holo-enzyme on Sepharose 4B

For details see the text.

Contents of incubation medium	Holoenzyme activity (-biotin) (munits/mg of protein)	Holo- plus apo-enzyme activity (+biotin) (munits/mg of protein)	Net apoenzyme (munits/mg of protein)
Fraction X	2.72	2.83	0.11
Fraction Y	0.72	0.72	Nil
Holoenzyme synthetase	Nil	Nil	Nil
Fraction X +holoenzyme synthetase	3.05	3.15	0.10
Fraction Y +holoenzyme synthetase	0.75	6.24	5.49

Fig. 2. Sepharose 4B chromatography of the apoenzyme preparation

A column (2.5cm×85cm) of Sepharose 4B was used. ●, Apoenzyme preparation, under polymeric conditions, was eluted with buffer C and fractions (3 ml) were collected for acetyl CoA carboxylase assay in the presence of biotin and holoenzyme synthetase; ▲, ^{14}C -labelled holoenzyme, under protomeric conditions, was eluted with buffer B. Fractions (3 ml) were collected and radioactivity was determined; ■, ^{14}C -labelled holoenzyme, under polymeric conditions, was eluted with buffer C. Fractions (3 ml) were collected and radioactivity was determined.

(mol.wt. $>4 \times 10^6$). The rats used in this experiment were in a moderate state of biotin deficiency (4 weeks on the avidin diet) so that the adipose tissue contained both the holo- and apo-enzymes. This was useful as a marker to identify the locations of the protomeric and polymeric forms of the enzyme in the column effluent (Fig. 1). Thus at a pH of 8.2 and in the absence of tricarboxylic acid effectors like citrate the enzyme was eluted from the column as a single peak (Y). Under conditions favouring aggregation, pH 6.9 and 10mM-citrate, there was a shift in the elution profile with the appearance of a high-molecular-weight peak (X). The enzyme preparation was fractionated on Sepharose 4B under conditions favouring aggregation and fractions corresponding to locations of the polymeric and

protomeric peaks respectively were pooled. Acetyl-CoA carboxylase activity was assayed in the pooled fractions X and Y (Fig. 1) both in the presence and in the absence of biotin and holoenzyme synthetase. The apoenzyme activity was calculated from the difference. The results are given in Table 1. The fact that in the presence of biotin and holoenzyme synthetase the carboxylase activity of pooled fraction Y increased almost ninefold with no increase at all in fraction X indicates that the apoenzyme is eluted from the column almost entirely along with the protomeric form of holo-(acetyl-CoA carboxylase). Thus under conditions that favour holoenzyme aggregation, the apoenzyme does not aggregate.

To confirm this, apo-(acetyl-CoA carboxylase) completely free from the holoenzyme was prepared by Sepharose-avidin affinity chromatography from the epididymal adipose tissue of rats in an advanced state of biotin deficiency (6 weeks on the avidin diet) so that the holoenzyme was further depleted. It has been shown earlier (Desjardins & Dakshinamurti, 1971b) that the apoenzyme is not depleted under these conditions. The apoenzyme was converted *in vitro* into the holoenzyme by incorporating D(+)-[carbonyl- ^{14}C]biotin. The radioactive holoenzyme, under conditions favouring the protomeric form, was eluted from the column of Sepharose 4B precisely at the position of the apoenzyme (Fig. 2). The shift in the elution profile of the labelled holoenzyme to the higher-molecular-weight region under conditions favouring aggregation, indicates that this transition is possible only in the presence of the prosthetic biotinyl group.

Discussion

The role of tricarboxylic acid effectors in activation of acetyl-CoA carboxylase by favouring the transition of the protomeric to the polymeric enzyme is now well established. The results presented here show that apo-(acetyl-CoA carboxylase) is not capable of aggregation to the polymeric state. This and the report of Moss & Lane (1972) that avidin cannot bind to holo-(acetyl-CoA carboxylase) in the aggregated form show that the biotinyl prosthetic group is the

site of the effector-mediated protomer-polymer transition of the enzyme.

Our earlier work (Dakshinamurti & Desjardins, 1968, 1969) and that of Jacobs *et al.* (1970) have demonstrated that the catalytic efficiency of acetyl-CoA carboxylase is regulated by the ratio of holo-enzyme to apoenzyme in tissues of biotin-deficient rats. Ryder (1972) has shown that in chick liver the apoenzyme is synthesized in response to hatching and that the active holoenzyme synthesis is provoked by feeding. It is possible that even in normal animals the conversion of the apo- into holo-enzyme might be a site of regulation in addition to the protomer-polymer transition of holo-(acetyl-CoA carboxylase).

Acetyl-CoA carboxylase apo- and holo-enzymes are not separated by the usual methods of enzyme purification. The results presented here show that under conditions favouring holoenzyme aggregation the apoenzyme can essentially be separated from the polymeric holoenzyme. This, in association with the previously reported (Landman & Dakshinamurti, 1973) Sepharose-avidin affinity chromatography, offers an effective method of preparing large amounts of apo-(acetyl-CoA carboxylase).

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