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# The Conversion of Acetyl-CoA Apocarboxylase into the Active Holocarboxylase by Acetyl-CoA Holocarboxylase Synthetase

by

Amiram Dan Landman

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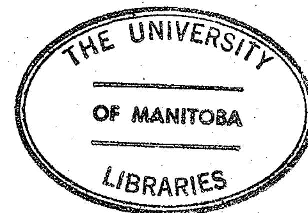

# "THE CONVERSION OF ACETYL-CoA APOCARBOXYLASE INTO THE ACTIVE HOLOCARBOXYLASE BY ACETYL-CoA HOLOCARBOXYLASE SYNTHETASE"

by

AMIRAM DAN LANDMAN

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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To my parents,

who dedicated themselves to my career

and to my wife Judith and daughter Shirley

who helped me along.

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## ABSTRACT

The conversion of acetyl-CoA apocarboxylase into the active holocarboxylase by acetyl-CoA holocarboxylase synthetase

By Amiram Dan Landman

Acetyl-CoA apocarboxylase and acetyl-CoA holocarboxylase are two proteins with virtually indistinguishable structures. Their separation,which is required for a study of the apo-holo conversion system, was obtained by affinity chromatography on a Sepharose-avidin column. The efficiency and specificity of this column in obtaining holo-free apocarboxylase preparations are demonstrated .

Acetyl-CoA holocarboxylase has a low intrinsic activity as a protomer. In the presence of citrate it aggregates; this is followed by a hundred fold increase in enzyme activity. Using techniques of selective sedimentation by a preparative ultracentrifuge and separation on Sepharose-4B, it was shown that the apocarboxylase which lacks enzymatic activity does not have the ability to aggregate, thus pointing to the microenvironment around biotin as the site which triggers the aggregation and activation simultanously.

In view of the difficulties in obtaining the apocarboxylase, the assay of acetyl-CoA holocarboxylase synthetase was tried by replacing the apocarboxylase with artificial substrates rich in lysine residues. As these were unsuccessful, a partial reaction of the holocarboxylase synthetase which involves the exchange of labelled $PP_i$ with ATP was devised for its assay.

"Biotin activating enzyme" was partially purified and some of its properties studied. Addition of the partially purified "Biotin activating enzyme" to cytosolic preparations of adipose tissue of biotin deficient rats did not enhance the rate of incorporation of biotin into endogenous acetyl-CoA apocarboxylase, which indicated that "Biotin activating enzyme" may be distinct from acetyl-CoA holocarboxylase synthetase.

A microbiological method has generally been used for the assay of biotin. Although the assay is accurate and reproducible it is time consuming. The radioisotope dilution assay for biotin described here has the sensitivity required to measure the levels of biotin found in biological material and is simple and rapid. The procedure was applied for the assay of biocytin, a biotin derivative which participates in the catabolism of biotin-containing-enzymes.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| AMP | : | Adenosine 5' monophosphate |
| ADP | : | Adenosine 5' diphosphate |
| ATP | : | Adenosine 5' triphosphate |
| Acetyl-CoA | : | Acetyl-Coenzyme A |
| ACHS | : | Acetyl-Coenzyme A holocarboxylase synthetase |
| ACAP | : | Acetyl-Coenzyme A apocarboxylase |
| BAE | : | Biotin activating enzyme |
| BSA | : | Bovine serum albumin |
| b-AMP | : | Biotinyl-AMP |
| CoA | : | Coenzyme A |
| DCC | : | Dicyclohexylcarbodiimide |
| DEAE | : | Diethylaminoethyl |
| EDTA | : | Ethylenediamine tetraacetic acid |
| E | : | Molar extinction coefficient |
| GSH | : | Glutathione (reduced) |
| HABA | : | 2(4' Hydroxyazobenzene) benzoic acid |
| I.U. | : | International units |
| $P_i$ | : | Inorganic phosphate |
| $PP_i$ | : | Pyrophosphate |
| PPO | : | 2,5 Diphenyloxazole |
| POPOP | : | 1,4-bis-2(5-phenyloxazolyl)-benzene |
| Tris | : | Tris (hydroxymethyl) amino methane |
| TCA | : | Trichloroacetic acid |

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## INTRODUCTION

Acetyl-CoA carboxylase is a biotin containing enzyme. The enzyme exists in both the apo and holo forms. The apoenzyme has no catalytic activity. The conversion of acetyl-CoA apocarboxylase to the holoenzyme is catalyzed by an enzyme, acetyl-CoA holocarboxylase synthetase. The purpose of this thesis was to study the interaction between acetyl-CoA holocarboxylase synthetase and the apocarboxylase, as well as to investigate further the relationship that biotin has with acetyl-CoA carboxylase.

Objectives: The first objective was to investigate rat liver acetyl-CoA holocarboxylase synthetase. In a series of reports Dakshinamurti and Desjardins (1969, 1970, 1971) have reported on the *in vivo* and *in vitro* conversion of acetyl-CoA apocarboxylase to the holoenzyme form in rat liver and adipose tissue. In their study they utilized an assay system which was encumbered by the presence of substantial holocarboxylase activity. Methods were developed in this thesis for an improved assay of acetyl-CoA holocarboxylase synthetase activity by the removal of this endogenous acetyl-CoA holocarboxylase by affinity chromatography. In addition, attempts were made to measure acetyl-CoA carboxylase synthetase in pig and rat liver using the colorimetric assay described by Coon et al. (1964). In their assay the formation of biotinyl-AMP was measured colorimetrically after its conversion to biotinylhydroxamate.

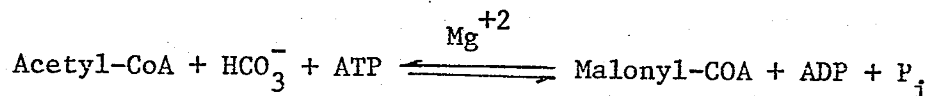
The second objective of the thesis was to investigate the role of the covalently bound biotin on acetyl-CoA carboxylase in the aggregation and disaggregation of the enzyme.

Organization of the thesis: The thesis is divided into two major sections. The first section presents the background information for this thesis and takes the form of a literature review. The next section is divided into five chapters. Each chapter contains appropriate introduction, experimental methodology, results and discussion. The first chapter deals with the isolation of acetyl-CoA apocarboxylase free of holocarboxylase activity. The next chapter describes the investigation into the role of biotin in the aggregation of the holoenzyme. The next two chapters describe attempts to improve the methodology for the assay of acetyl-CoA holocarboxylase synthetase and in the last chapter a radioisotope dilution assay for biotin is described.

## LITERATURE REVIEW

### A. Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase was initially recognized by Wakil and coworkers (1958) as fraction $R_1$ which in the presence of the complementary fraction $R_2$ catalyzed the formation of fatty acids in the soluble fraction of crude pigeon liver extracts. Fraction $R_2$ was later identified as the fatty acid synthetase complex (Wakil and Ganguly, 1959). Further investigation into the nature and role of fraction $R_1$ showed that bicarbonate was required for its activity and malonyl-CoA was identified as one of the products (Formica and Brady, 1959). It has now been shown (Wakil, 1961) that fraction $R_1$ catalyzes the following reaction:



$$\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \xrightleftharpoons{Mg^{+2}} \text{Malonyl-COA} + \text{ADP} + P_i$$

Subsequently, acetyl-CoA carboxylase was identified as a biotin containing enzyme (Wakil and Gibson, 1960).

Brady and Gurin (1952) were the first to show that certain tricarboxylic acid cycle intermediates such as citrate stimulated fatty acid synthesis. Martin and Vagelos (1962, 1963) showed that citrate stimulated fatty acid synthesis at the acetyl-CoA carboxylase step. They also showed that activation of acetyl-CoA carboxylase by citrate resulted in an aggregation of the enzyme to a heavier sedimenting form. Lane and coworkers (1968, 1968a) have confirmed and extended the findings of Martin and Vagelos by using avian liver acetyl-CoA carboxylase purified to homogeneity. Subsequent studies by numerous investigators (Moss and Lane, 1971; Lane, Moss and Polakis 1974; Numa and Yamashita, 1974) have elucidated

the structure, function and the reaction mechanism of this enzyme. The acetyl-CoA carboxylase reaction was shown to be regulated by a variety of biochemical and physiological determinants and is considered by many as the rate limiting and regulatory step of fatty acid synthesis.

i. Physical properties of acetyl-CoA carboxylase

Acetyl-CoA carboxylase has been purified to homogeneity from chicken liver (Lane et al. 1968, 1968a), bovine adipose tissue (Moss et al. 1972) and rat liver (Inoue and Lowenstein, 1972). The enzyme from these various sources has similar physico-chemical properties. The enzyme exists in both the protomeric and the polymeric forms,and the equilibrium between these forms is affected by various factors such as protein concentration, pH, citrate, malonyl-CoA, phosphate etc. The protomeric form has $S_{20,w}$ of 13-15 which corresponds to a molecular weight of 410,000-550,000 daltons while the polymeric form has a molecular weight of 4-5 million daltons.

The substructure of the rat liver enzyme was analyzed by Inoue and Lowenstein (1972). Examination of rat liver acetyl-CoA carboxylase on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate dissociated the enzyme into two subunits of 215,000 daltons which could further be dissociated into nonidentical polypeptide chains of 118,000 and 125,000 daltons by prolonged and repeated treatment of the enzyme with sodium dodecyl sulfate at an elevated temperature. These investigators concluded that the enzyme contains pairs of unlike polypeptide chains which remain associated to a considerable degree in sodium dodecyl sulfate at room temperature, but which can be separated after more drastic treatment. Guchhait, Zwergel and Lane (1974) have demonstrated

that chicken liver acetyl-CoA carboxylase in the presence of 6 M urea and 0.1% sodium dodecyl sulfate dissociates into three polypeptide chains of 117,000, 129,000 and 139,000 daltons. The ratio of these chains was found to be 2:1:1 respectively. The difference in the structures of the chicken and rat carboxylases is reflected in their biotin content. While the rat liver enzyme is reported to contain two biotin molecules, presumably one for each 215,000 dalton subunit, the chicken acetyl-CoA carboxylase is reported to possess only one molecule of biotin per protomer which is presumeably attached to the 117,000 dalton polypeptide chain. More data is required to clarify whether the differences in structure and biotin content of the chicken and rat carboxylases as reported by Lane's and Lowenstein's groups is inherent to these species, or whether it is a result of different technical procedures.

ii. Effect of citrate on acetyl-CoA carboxylase

Citrate is an allosteric effector of the Vmax type (Numa, Ringelmann and Lynen, 1965). The activity of the protomeric form of the enzyme from various sources is within the range of 0.15-0.30 units/mg (one unit is defined as an activity which will catalyze the formation of malonyl-CoA in a rate of 1 μM/min.), while in the presence of citrate the enzyme aggregates and the activity is increased to 10-15 units/mg (Moss and Lane, 1971). Rat liver and rat adipose tissue are activated by citrate only after incubation at 37°C for 30-60 minutes (Dakshinamurti and Desjardins, 1969; Desjardins and Dakshinamurti, 1970). However, under appropriate conditions and in the presence of citrate or isocitrate, chicken acetyl-CoA carboxylase will aggregate within 10 seconds. Moss and Lane (1972) investigated the nature and interrelationship between the

activation and aggregation of the chicken enzyme by cooling the enzyme to 2°C, a temperature at which both processes were slowed to a considerable extent and therefore could be studied with relative ease. The enzyme was incubated in presence of phosphate in order to keep it in its aggregated state (this however did not increase the enzymic activity), and then transferred into a medium which contained the components of the assay mixture with the exception of citrate. Disaggregation of the enzyme was followed by measuring viscosity. The rate of disaggregation paralleled the rate of loss in enzyme activity. From this study it was concluded that the aggregates are "open structures" in which the number of protomers on the oligomer is not defined, and may vary depending on the pH, protein concentration and other parameters defined by Gregolin et al. (1968, 1968a), and that the enzymic activity is possibly a function of the size of the structures. It has been suggested that the "raison d'être" for the changes in the supraquaternary structure is the intimate coordination between acetyl-CoA carboxylase and other lipogenic enzymes, mainly fatty acid synthetase. Thus the macromolecular changes in the enzyme during aggregation may provide a delicate matrix for the stereospecific assembly of the fatty acid synthetase (Moss and Lane 1971, 1972).

Avidin is a glycoprotein of a molecular weight of 68,000 which exhibits a very strong affinity to free or protein-bound biotin. The dissociation constant between the two components $K_D = 10^{-15}$ reflects this affinity (Green, 1963; 1965). The glycoprotein inhibits acetyl-CoA carboxylase by irreversibly binding with the prosthetic group biotin. Moss and Lane (1972) have shown that this inhibition will occur only in the absence of citrate, but not in the presence of the activator. This

led to the conclusion that the structural changes induced by citrate involve the microenvironment surrounding the active site which will protect the prosthetic group from the inhibitor. Apparently aggregation per se does not provide this protection, since when the polymerization is induced by tricarballylate which is not coupled with increased enzyme activity, the rate of binding of avidin to the biotin is similar to the binding under conditions favoring its protomeric form.

Further evidence in regard to the structural changes induced by citrate come from model experiments on the carboxylation of free biotin (Lane, Moss and Polakis, 1974). When acetyl-CoA carboxylase was incubated with biotin and the system supplemented with $Mg^{++}$, ATP and $HCO_3^-$, the free biotin was carboxylated. However, on addition of citrate to this mixture the Km for the biotin was highly increased. This implied that the changes induced by the activator help to orient the enzyme bound biotin in a position which will favor its carboxylation but will hinder the free biotin from approaching the carboxylation site.

Moss and Lane (1972) have demonstrated that phosphate which is an aggregator is also capable of activation but that the activity is lost rapidly and can be detected only by specially designed experiments. It is generally accepted that phosphate and tricarballylate are aggregators while citrate and isocitrate are "true activators." It was postulated that the carboxylation of the biotin at the active site, causes structural strain near the active site. This strain causes further structural changes in the quartenary structure which will result in the disaggregation of the polymer. Only the "true activators" are capable of withstanding this strain, keeping the catalytic site in its