THE FORMATION OF MALONYL-ENZYME AND ITS CONVERSION TO FATTY ACIDS AND β-HYDROXY, β-METHYL GLUTARYL COENZYME A*

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Received May 16, 1963

Previous reports from this laboratory (Brodie, Wasson and Porter, 1962; Brodie, Wasson and Porter, 1963) demonstrated that malonyl CoA is a substrate for the biosynthesis of mevalonic and long chain fatty acids by a partially purified avian liver enzyme system. These studies also showed that neither acetoacetyl CoA nor β-hydroxy, β-methyl glutaryl coenzyme A (HMG-CoA) are intermediates in the formation of mevalonic acid. As a result of these findings it was concluded that acetoacetyl-enzyme and HMG-enzyme are intermediates in the formation of mevalonic acid and that acetoacetyl-enzyme is an intermediate in the formation of fatty acids. Further support for this conclusion is found in the present communication in which evidence is presented for the formation of malonyl-enzyme and for its conversion to HMGCoA and long chain fatty acids. The importance of HMGCoA in the present studies is found in the fact that this compound is formed, in the absence of TPNH, from the true intermediate in mevalonic acid biosynthesis. Its

* This investigation was supported by research grants A-1383 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U.S. Public Health Service and 61 G 7 from the American Heart Association.
The authors express their appreciation to Mr. E.A. Steberl for the enzyme preparations used in these studies.

presence therefore reflects the conversion of substrate to intermediates in the biosynthesis of sterols.

The binding of malonate from malonyl CoA to enzyme protein in the absence of acetyl CoA is shown in Table I. Evidence that malonate is covalently bound to protein is found in the fact that only alkaline hydrolysis could remove the radioactivity from the protein. Furthermore, the conditions required for the removal of malonate from the protein are far more drastic than those required to split malonyl CoA. Complete removal of malonate from the enzyme after acid denaturation requires heating at 70° in the presence of 1 N KOH for 2 hours. After one hour at 38° in 1 N KOH only 25% of the malona is liberated from the protein. It is quite unlikely, therefore, that any of the malonyl moiety bound to protein after acid precipitation and washing represents malonyl CoA.

The data on inhibition of the binding of malonate to enzyme, which are also shown in Table I, indicate that the malonyl binding site on the enzyme is a sulfhydryl group. This site has a high sensitivity to N-ethyl maleimide and little sensitivity to alkylation by iodoacetamide. Malonyl CoA transacylase activity by this enzyme, with pantetheine as acceptor, is also strongly inhibited by N-ethyl maleimide and insensitive to iodoacetamide. This finding is very similar to the findings of Alberts, et al. (1963) on the inhibition of malonyl CoA transacylase activity in an enzyme preparation obtained from C. kluyveri. Thus these results, plus other evidence presented earlier (Lynen, 1961, Brodie, Wasson and Porter, 1963), support the concept that enzyme sulfhydryl attacks malonyl CoA with the formation of malonyl-S-enzyme and the displacement of $CoAS^-$.

The direct isolation of malonyl-enzyme by the following procedure, and its conversion to HMGCoA and fatty acids, offers the first direct proof that

TABLE I

CONVERSION OF 2-C^{14}-MALONYL CoA TO 2-C^{14}-MALONYL-ENZYME

IN THE PRESENCE AND ABSENCE OF INHIBITORS

ADDITION		C^{14}-Labeled Protein c/min/mg	Inhibition %
None		804	
N-Ethyl maleimide,	1×10^{-5} M	637	21
	1×10^{-4} M	61	92
	1×10^{-3} M	29	97
Iodoacetamide,	1×10^{-3} M	625	22
	5×10^{-3} M	525	35
	9×10^{-3} M	530	35

12.6 mg of enzyme protein were preincubated with inhibitor for 30 minutes at 38°. 2-C^{14}-Malonyl CoA (117,000 c/min and 79 mμmoles) was then added. After one minute of incubation the protein was precipitated with perchloric acid, washed 3 times with 0.2 M acetic acid and finally dissolved in dilute base for a determination of radioactivity by liquid scintillation counting. Protein of the precipitate was determined by the biuret method of Gornall, et al., (1949).

this compound is an intermediate in fatty acid and sterol synthesis. Malonyl-enzyme is obtained on incubation of 2-C^{14}-malonyl CoA with enzyme for one minute. The labeled protein is then precipitated with solid ammonium sulfate. The precipitate is dissolved in dilute phosphate buffer, pH 7.0, and the ammonium sulfate precipitation is repeated. The second precipitate is dissolved in 0.1 M phosphate buffer, pH 7.0, and passed through a column of Sephadex G-25 previously equilibrated with 0.1 M phosphate buffer, pH 7.0. The eluted labeled protein is then free of C^{14}-labeled malonyl CoA.

Incubation of the labeled protein with TPNH and large pools of both acetyl and malonyl CoA resulted in the incorporation of radioactivity into long chain

fatty acids, Table II. Additional proof of the absence of C^{14}-malonyl CoA in association with protein is found in these studies. It can be seen from Table II that the addition of acetyl CoA alone did not result in an appreciable incorporation of label into fatty acids. The addition of both acetyl and malonyl CoA was necessary for maximal incorporation. If the radioactivity associated with the protein had been malonyl CoA, maximal incorporation would have been achieved with acetyl CoA alone, and the addition of a pool of non radioactive malonyl CoA would have decreased incorporation.

TABLE II

INCORPORATION OF C^{14} FROM C^{14}-LABELED ENZYME INTO FATTY ACIDS

Substrates	Radioactivity in Fatty Acids c/min
1. C^{14}-enzyme	75
2. C^{14}-enzyme + acetyl CoA, .05 μmole	110
3. C^{14}-enzyme + acetyl CoA, .05 μmole + malonyl CoA, 0.25 μmole	740

Each incubation mixture contained: C^{14}-enzyme (2450 c/min, 1.6 mμmoles and 2.2 mg of protein); mercaptoethanol, 10 μmoles; TPNH, 2 μmoles; phosphate buffer, pH 7.0, 54 μmoles; in a total volume of 1.0 ml. Samples were incubated at 38° for 1 hour.

When larger quantities of labeled enzyme were incubated in the presence of acetyl and malonyl CoA, and in the absence of TPNH, HMGCoA was formed. The latter compound was cleaved by alkaline hydrolysis and then identified by paper chromatography. It migrated with authentic HMG at an R_f of 0.56 in an ammonia-isobutyric acid system (Brodie and Porter, 1960). The radioactive

component was further identified as HMG through methylation in the presence of carrier HMG, followed by gas-liquid chromatography on a butanediol succinate column at 188°, Figure 1. The effluent containing the dimethyl HMG was trapped on glass wool in a dry-ice acetone bath and then counted in a Packard Tri-Carb liquid scintillation spectrometer. Identification of HMG was further confirmed by recrystallization of the di-p-bromophenacyl ester to constant specific radioactivity.

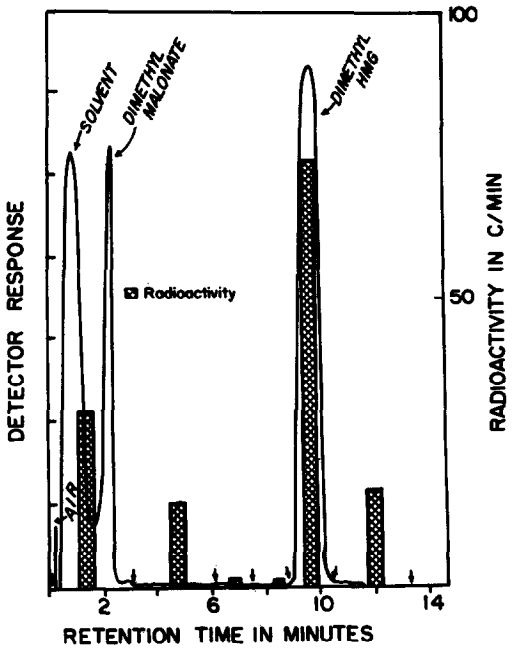

Figure 1. Association of radioactivity of product formed from Malonyl-enzyme with Dimethyl HMG. Chromatographic separation was made on a 6' x 6 mm column of 20% butanediol succinate on acid washed Chromosorb W at 188° with an argon flow rate of 100 ml/min.

The data of Table II show that approximately 30% of the radioactivity associated with protein was incorporated into fatty acids. Since it was expected that a greater conversion would be obtained, an assay was made to determine whether all of the radioactivity associated with the protein was malonyl-enzyme.

The protein eluted from the Sephadex was precipitated with perchloric acid and washed with 0.2 N acetic acid. Approximately 60% of the radioactivity was obtained in the supernatant solution. These washes were combined and lyophilized to dryness, at pH 1. The residue was then extracted with acetone, the extract was methylated, and the products were subjected to gas-liquid chromatography. All of the radioactivity remaining after lyophilization was extracted with acetone. The bulk of the radioactivity was accounted for as dimethyl malonate on gas-liquid chromatography, but small amounts of unidentified low molecular weight components were also present. The fact that an alkaline treatment was not needed to release the radioactive compounds associated with the protein, and the fact that the counts could be directly methylated as proven by gas-liquid chromatography, demonstrates conclusively that the associated counts were neither acyl-SCoA nor acyl-S-enzyme. It is probable that the free malonic acid originated from malonyl-enzyme through deacylase activity during passage through Sephadex and that it was maintained in close proximity to the protein through ionic interaction.

The radioactive compound remaining on the protein after acid treatment was hydrolyzed with base. Independent determinations by both paper and gas-liquid chromatography for the identity of the component showed that it was malonate. Therefore, correction of the counts in Table II for those present as associated free acid, results in a much higher conversion of radioactivity from C^{14}-malonyl-enzyme to long chain fatty acids (about 75%).

In the course of these studies we have also demonstrated the independent binding of acetate from acetyl-CoA to the enzyme and the formation of enzyme bound acetoacetate from malonyl-enzyme. All data available at the present time is consistent with the proposal (Brodie, Wasson and Porter, 1963) that

acetoacetyl enzyme is the key intermediate in the synthesis of mevalonic and fatty acids by this enzyme system.

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