CARBOXYLATION OF ACETONYLDETHIO-COENZYME A BY ACETYL COENZYME A CARBOXYLASE

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Received 10 May 1978

1. Introduction

Acetonyldethio-coenzyme A has been synthesized [1] together with its 2′-phosphate isomer as a unique analogue of acetyl-CoA; in this analogue, hereafter referred to as acetono-CoA, the sulfur is replaced by methylene. Acetono-CoA was shown to exhibit strong competitive inhibition of citrate synthase, which mediates the formation of citrate from acetyl-CoA and oxaloacetate [1]. The present investigation was designed to examine the effect of acetono-CoA on acetyl-CoA carboxylase (acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2), which catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, ADP and orthophosphate [2] and plays a critical role in the regulation of fatty acid biosynthesis [3]. The results obtained have demonstrated that acetono-CoA serves as a substrate of acetyl-CoA carboxylase from rat liver; the apparent K_m value for acetono-CoA is approximately five-times as high as that for acetyl-CoA, while the V_{max} value for acetono-CoA is approximately one-seventh that for acetyl-CoA. Available evidence indicates that the terminal methyl group of acetono-CoA is carboxylated as is the case with acetyl-CoA.

2. Materials and methods

2.1. *Enzymes, chemicals and determinations*

Pure acetyl-CoA carboxylase from rat liver was prepared by the method in [4] as modified in [5]. Yeast fatty acid synthetase was a generous gift of Professor F. Lynen and Dr F. Wieland. CoA, pyruvate kinase and lactate dehydrogenase were purchased from Boehringer (Mannheim). $NaH^{14}CO_3$ was a product of the Radiochemical Centre (Amersham). Acetono-CoA was synthesized as in [1] and was determined spectrophotometrically by assuming a molar absorbance at 260 nm of 16 400 $M^{-1}cm^{-1}$ [6]. Acetyl-CoA was prepared by the method in [7] and determined as in [8]. Protein was determined by the method in [9] with bovine serum albumin (Sigma, St Louis, MO) as the standard. All other chemicals were of analytical grade.

2.2. *Assays of acetyl-CoA carboxylase*

Acetyl-CoA carboxylase activity was determined at 37°C either by the isotopic assay with $H^{14}CO_3^-$ or by the spectrophotometric assay with the aid of pyruvate kinase and lactate dehydrogenase. For the isotopic assay, the enzyme was preincubated at 37°C for 15 min as in [4], except that $MgCl_2$ was omitted from the preincubation mixture, and the preincubated enzyme was further incubated for 20 min in the reaction mixture [4], except that the total volume of the mixture was reduced to 0.4 ml and that, when indicated, acetyl-CoA was replaced by acetono-CoA. The reaction was terminated by rapid cooling in iced water. After the addition of 2 drops of 5% *n*-octanol in methanol, CO_2 was bubbled through the mixture at 0°C for 1 h to remove the excess $H^{14}CO_3^-$ [10]. The mixture was then assayed for radioactivity in the scintillator solution [11] with a liquid scintillation spectrometer. Alternatively, the reaction was terminated by the addition of HCl at a final conc. 1 M. The mixture was exposed to reduced pressure at room temperature for 5 h and then neutralized with KOH. The radioactivity of the 'acid-stable' material was determined as above.

The spectrophotometric assay was carried out as in [4] with the following exceptions. The reaction mixture contained 1.88 mM ATP and, when indicated, acetono-CoA at specified concentrations instead of acetyl-CoA. A mixture containing all components except acetyl-CoA (or acetono-CoA) was preincubated at 37°C for 15 min, and the reaction was initiated by the addition of acetyl-CoA (or acetono-CoA). Purified rat liver acetyl-CoA carboxylase, unlike cruder enzyme preparations [12], exhibited a full activity even when the reaction was started with acetyl-CoA (or acetono-CoA).

2.3. *Identification of the carboxylation product of acetono-CoA*

The reaction mixture for the isotopic assay was scaled up 5-fold, containing $H^{14}CO_3^-$ of higher specific radioactivity (5 μCi/μmol) and 8.5 μg acetyl-CoA carboxylase. Other experimental conditions were as above. After the bubbling of CO_2 for 2 h, the mixture was concentrated in vacuo to ~ 0.5 ml and adjusted to pH 6 by the addition of 2 M HCl. This was applied to a Sephadex G-10 column (1.8 × 13 cm), and the column was eluted with distilled water. The radioactive material eluted (1.9×10^5 cpm) was applied to a DEAE-cellulose column (1.8 × 10 cm) pre-equilibrated with 3 mM HCl. The column was eluted with a linear gradient of 0–0.15 M LiCl in 3 mM HCl (total vol. 1 litre) [13]. The fractions containing the labelled material (1.2×10^5 cpm) were collected, concentrated and subjected to gel filtration on Sephadex G-10 as above. The carboxylation product in 90% aqueous methanol was esterified by reaction with a large excess of diazomethane in diethyl ether. The esterified product was taken to dryness in vacuo and dissolved in 2 ml trifluoroacetic acid. This was mixed with 25 μl conc. H_2SO_4 and 0.4 ml 1.4 M hydrazoic acid in chloroform, and the mixture was heated at 50°C for 5 h. The solvent was removed in vacuo, and the remaining material (8.9×10^4 cpm), dissolved in 2 ml 6 M HCl, was hydrolyzed in an evacuated sealed tube at 110°C for 24 h. After removal of the solvent in vacuo, the residue was dissolved in water (4.6×10^4 cpm). An aliquot (1×10^4 cpm) of the hydrolysis product, mixed with authentic glycine and α,γ-diaminobutyric acid, was treated with benzoyl chloride in the presence of 1 M $KHCO_3$. After acidification with 5 M HCl, the benzoyl derivatives were extracted with ethyl acetate, and the solvent was removed in vacuo. The remaining material, dissolved in methanol, was esterified by reaction with diazomethane (4×10^3 cpm). An aliquot of the esterified material was subjected to thin-layer chromatography on a silica gel 60 F_{254} plate (Merck, Darmstadt) either with diethyl ether or with chloroform/methanol (50/1, by vol.) as the developing solvent. Equal segments (1 cm wide) of the chromatogram were scraped and assayed for radioactivity as described above.

3. Results

It is evident from table 1 that acetono-CoA was carboxylated by rat liver acetyl-CoA carboxylase. This was demonstrated both by the isotopic assay with $H^{14}CO_3^-$ and by the spectrophotometric assay with the aid of pyruvate kinase and lactate dehydrogenase. The same cofactor requirement was observed for the carboxylation of acetono-CoA as for that of acetyl-CoA [2,12].

In the experiment represented in fig.1, the carboxylation rate was determined with varying concen-

Table 1
Cofactor requirement for the carboxylation of acetono-CoA by acetyl-CoA carboxylase

Expt.	System	Activity	
		$H^{14}CO_3^-$ fixed[a] (cpm)	NADH oxidized (ΔA_{334}/min)
1	Complete	634	
	ATP omitted	10	
	Mg^{2+} omitted	12	
	Acetono-CoA omitted	22	
	Enzyme omitted	10	
	Zero time (immediate termination)	10	
	Complete, but enzyme boiled[b]	6	
2	Complete		0.038
	ATP omitted		0
	Acetono-CoA omitted		0
	HCO_3^- omitted		0.002
	Citrate omitted[c]		0.006

[a] The excess $H^{14}CO_3^-$ was removed by CO_2 bubbling. Essentially identical results were obtained when 'acid-stable' radioactivity was measured

[b] The preincubated enzyme was heated in a boiling water bath for 3 min and then added to the reaction mixture

[c] Citrate was omitted from the preincubation mixture as well

Enzyme activity was determined with 0.42 mM acetono-CoA by the isotopic assay (expt. 1) or by the spectrophotometric assay (expt. 2) according to the procedure in section 2, except that the assay system was modified as indicated and that, in expt. 2, the reaction was initiated by the addition of the enzyme that was preincubated as in the case of the isotopic assay; the amount of the enzyme added was 0.55 μg (expt. 1) or 5.5 μg (expt. 2), and the specific radioactivity of $H^{14}CO_3^-$ used was 0.25 μCi/μmol

trations of acetono-CoA or acetyl-CoA. Lineweaver-Burk plots [14] have shown that the app. K_m values for acetono-CoA and acetyl-CoA are 96 μM and 20 μM, respectively, while the V_{max} values for acetono-CoA and acetyl-CoA are 1.0 μmol/min mg protein^{-1} and 6.8 μmol/min mg protein^{-1}, respectively.

Since acetono-CoA and its carboxylation product have no thioester linkage, they would not be utilized as substrates of fatty acid synthetase, which mediates the formation of long-chain fatty acids or their CoA-derivatives from acetyl-CoA, malonyl-CoA and NADPH [15]. In fact, acetono-CoA at a concentration of 0.105 mM or 0.21 mM neither functioned as a primer for the reaction catalyzed by yeast fatty acid synthetase nor inhibited this enzyme. If both acetyl-CoA and acetono-CoA were added to a composite assay mixture for the coupled acetyl-CoA carboxylase-fatty acid synthetase reactions, malonyl-CoA resulting from the carboxylation of acetyl-CoA, but not the carboxylation product of acetono-CoA, would be utilized by fatty acid synthetase. Therefore, the K_m value of acetyl-CoA carboxylase for acetono-CoA would be measured as an app. K_i value. In the experiment represented in fig.2, the rate of malonyl-CoA formation was determined with varying concentrations of acetyl-CoA in the presence or absence of acetono-CoA at different fixed concentrations. As shown by Lineweaver-Burk plots [14], competitive inhibition by acetono-CoA was observed with respect to acetyl-CoA. The apparent K_i value for acetono-

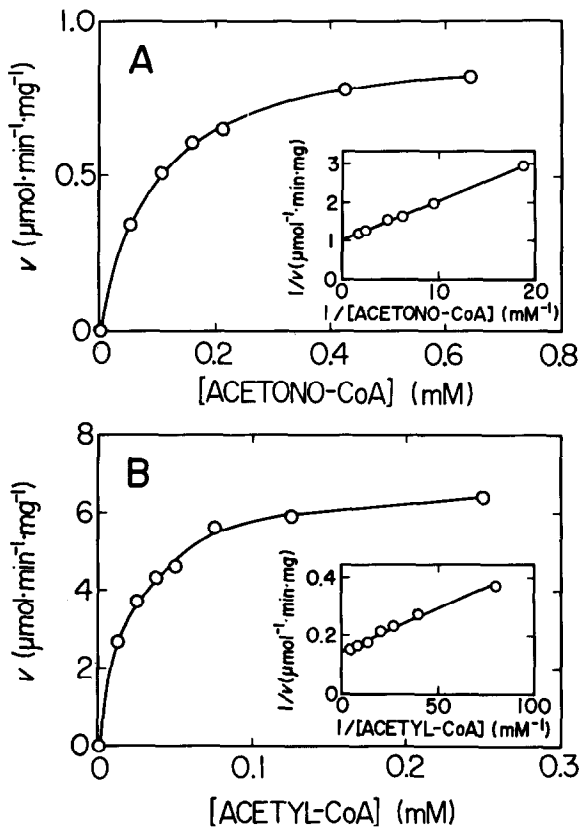
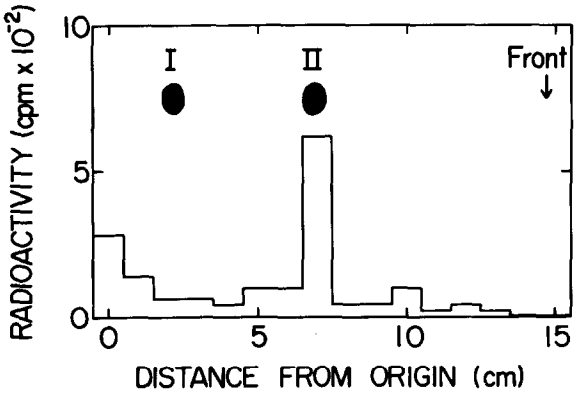


Fig.1. Effect of the concentration of acetono-CoA or acetyl-CoA on the carboxylation rate (v). Enzyme activity was determined by the spectrophotometric assay as in section 2, except that the concentration of acetono-CoA (A) or acetyl-CoA (B) was varied as indicated; the amount of the enzyme added was 5.5 μg. The inserted figures represent Lineweaver-Burk plots.



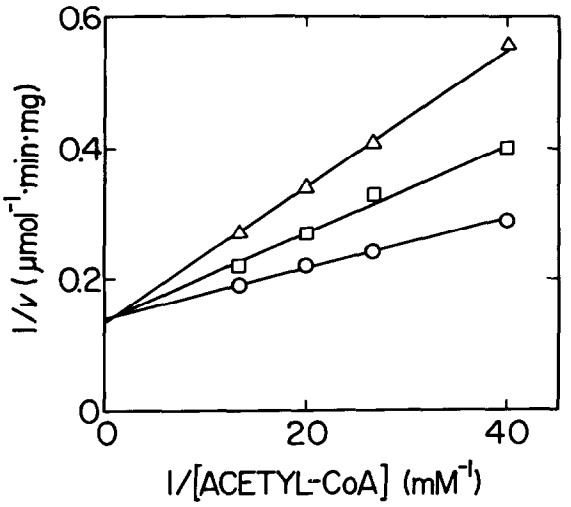


Fig.2. Effect of the concentration of acetono-CoA on the rate of carboxylation of acetyl-CoA (v) as examined by the coupled acetyl-CoA carboxylase-fatty acid synthetase system. The reaction mixture for the spectrophotometric assay in section 2 was modified as follows: Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate and NADH were replaced by 73 μg/ml fatty acid synthetase and 0.11 mM NADPH; the concentrations of acetyl-CoA and acetono-CoA were varied as indicated. The amount of acetyl-CoA carboxylase added was 5.5 μg. A mixture containing all components except NADPH and ATP was preincubated at 37°C for 15 min, and the reaction was initiated by the addition of NADPH and ATP. The oxidation of NADPH was followed at $A_{334\ nm}$. (○) No acetono-CoA; (□) 0.105 mM acetono-CoA; (△) 0.21 mM acetono-CoA.

CoA was calculated to be 120 μM, being in agreement with the K_m value for this compound.

There are two possible sites at which acetono-CoA can be carboxylated, i.e., the terminal methyl group and the methylene group neighboring the carbonyl. The products formed would be the γ- and α-substituted acetoacetic acid derivatives, respectively. If

Fig.3. Thin-layer chromatography of the amino acid derivative formed from the carboxylation product of acetono-CoA. For experimental details, see section 2. The developing solvent used was diethyl ether. Authentic standards, i.e., N^{α},N^{γ}-dibenzoyl-α,γ-diaminobutyric acid methyl ester (I) and N-benzoylglycine methyl ester (II), were located by ultraviolet irradiation.

these compounds were subjected to the Schmidt reaction, which represents a well-known method for the chemical synthesis of α-amino acids [16], and were then hydrolyzed, glycine or α,γ-diaminobutyric acid would be yielded. The carboxylation product formed with $H^{14}CO_3^-$ was esterified with diazomethane, and the methyl ester was subjected to the Schmidt reaction. The resulting material was hydrolyzed and converted to the benzoyl amino acid methyl ester. Analysis of this compound by thin-layer chromatography with diethyl ether as the developing solvent indicated the presence of the glycine derivative as a radioactive band but not of the α,γ-diaminobutyric acid derivative (fig.3). This was confirmed by thin-layer chromatography with chloroform/methanol (50/1, by vol.) as the developing solvent. Thus, it is concluded that the site of carboxylation is the terminal methyl group of acetono-CoA as is the case with acetyl-CoA.

4. Discussion

The present investigation has demonstrated that acetono-CoA serves as a substrate of acetyl-CoA carboxylase. The high K_m value and the low V_{max} value for acetono-CoA indicate that the enzyme has weaker affinity for this analogue than for acetyl-CoA owing to the replacement of the sulfur by methylene and that the methyl group attached to carbonyl is less reactive than that attached to the carbonyl of thioester.

Citrate synthase has been shown to be inhibited by acetylaminodethio-CoA as well as by acetono-CoA [1,17]. Likewise, phosphotransacetylase has been shown to be inhibited by acetylaminodethio-CoA [17]. In contrast, acetyl-CoA carboxylase utilizes acetono-CoA as a substrate. This difference can be accounted for by the fact that the cleavage of the thioester bond is not involved in the acetyl-CoA carboxylase reaction.

Acknowledgements

This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, the Mitsubishi Foundation, the Foundation for the Promotion of Research on Medicinal Resources, the Japanese Foundation of Metabolism and Diseases, and the Japanese Medical Association.

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