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Mechanism of action of β-oxoacyl-CoA thiolase from rat liver cytosol

Direct evidence for the order of addition of the two acetyl-CoA molecules during the formation of acetoacetyl-CoA

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Single-turnover enzyme reactions were employed with β-oxoacyl-CoA thiolase purified from rat liver cytosol to determine the order of binding of the two acetyl-CoA molecules to the enzyme during the formation of acetoacetyl-CoA. Equimolar quantities of [1-^{14}C]acetyl-CoA and enzyme were mixed initially in a rapid mixing device and the reaction was quenched by addition of an excess of unlabelled acetyl-CoA. Degradation of the resulting acetoacetyl-CoA revealed that the larger proportion of the radioactivity was in C-3. In the converse experiment, in which unlabelled acetyl-CoA was mixed with enzyme and the reaction was quenched with [1-^{14}C]acetyl-CoA, radioactivity was incorporated preferentially into C-1. Similar results were obtained when [^{14}C]-acetyl–enzyme complex isolated by gel filtration was reacted with unlabelled acetyl-CoA, the radioactivity appearing largely in C-3. These findings lead to the conclusion that of the two molecules of acetyl-CoA that are bound by the enzyme and converted into acetoacetyl-CoA, it is the one giving rise to C-3 and -4 that is bound initially to the enzyme in the form of the acetyl–enzyme intermediate complex.

β-Oxoacyl-CoA thiolase (acetyl-CoA:acetyl-CoA *C*-acetyltransferase; EC 2.3.1.9) from mammalian liver cytoplasm catalyses the reaction between two molecules of acetyl-CoA to give acetoacetyl-CoA (Middleton, 1971, 1973, 1974):

$$CH_3COSCoA + CH_3COSCoA \rightleftharpoons CH_3COCH_2COSCoA + CoASH$$

The reaction is the first committed stage in the biosynthesis of mevalonic acid, the direct precursor of cholesterol and other sterols. Although the reaction equilibrium strongly favours acetoacetyl-CoA cleavage ($K_{eq.} = 2 \times 10^{-5}$ at pH 8.2; Stern, 1956), the net flux of acetyl-CoA into acetoacetyl-CoA and thence to mevalonic acid is made possible by the efficient conversion of the acetoacetyl-CoA into hydroxymethylglutaryl-CoA.

The cytoplasmic enzyme has been isolated in homogeneous form by Middleton (1974) and shown to be a tetramer with a subunit mol.wt. of 40000. β-Oxoacyl-CoA thiolase is also found in mitochondria where two distinct types exist, one involved predominantly with β-oxidation and having a specificity for long-chain β-oxo thioesters and the other with a short-chain-length specificity, which is concerned with the formation of ketone bodies (Huth *et al.*, 1975).

The interest in the catalytic mechanism of β-oxoacyl thiolase stemmed initially from the work of Lynen (1953) and Gehring & Harris (1968), who highlighted the importance of a reactive cysteine residue at the active site from a study of several alkylating agents. Direct evidence in support of a mechanism involving the formation of a covalent thioester link between this reactive cysteine and one of the two acyl moieties was first provided when a [^{14}C]acetyl–enzyme complex, isolated after incubation of mitchondrial thiolase with [^{14}C]acetyl-CoA, was shown to contain [^{14}-C]acetylcysteine (Gehring & Harris, 1968, 1970). The enzyme exhibits Ping Pong kinetics (Middleton, 1974; Huth *et al.*, 1975) consistent with the intermediacy of an acyl–enzyme complex. The stereochemical course of the reaction has been elucidated by Willadsen & Eggerer (1975), who established that, in the direction of acetoacetyl-CoA synthesis, the formation of the methylene group at C-2 occurs with inversion of configuration at the methyl group of acetyl-CoA.

The mechanism of action of β-oxoacyl-CoA thiolase generally considered to be operative involves the initial binding of acetyl-CoA to the

enzyme followed by the release of CoASH and the establishment of an acyl–enzyme complex. Reaction of the second molecule of acetyl-CoA follows, with the concomitant breakage of the acetyl–enzyme thiol ester bond and formation of acetoacetyl-CoA. This mechanism has been arrived at largely by analogy with β-oxoacyl-CoA thiolases, which participate in the reactions of long-chain β-oxo thioesters.

The reaction of mitochondrial β-oxoacyl-CoA thiolase is unique amongst thiolases in that two molecules of the same substrate, namely acetyl-CoA, are involved. Investigation of the order of substrate binding during the synthesis of acetoacetyl-CoA, and release of acetyl-CoA during thiolysis using steady-state-kinetics methods is not therefore possible. In the present paper we describe an alternative approach to the study of the mechanism of action of cytoplasmic β-oxoacyl-CoA thiolase from rat liver using a single-turnover enzyme reaction procedure. This technique has enabled us to determine directly the order of addition of the two molecules of acetyl-CoA to the enzyme during the biosynthesis of acetoacetyl-CoA.

Experimental

Materials

[1-^{14}C]Acetyl-CoA and $NaH^{14}CO_3$ were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. CoA and lithium acetoacetate were purchased from Sigma Chemical Co. Cellulose phosphate and DEAE-cellulose were obtained from Whatman, Maidstone, Kent, U.K. Acetic anhydride, diketene (vinyl aceto-β-lactone) semicarbazide hydrochloride and all other chemicals and reagents were purchased from BDH, Poole, Dorset, U.K.

Methods

Preparation of acyl-CoA derivatives. Acetyl-CoA was prepared as follows. CoA (30mg) was dissolved in 6ml of water at 0°C in a 50ml round-bottomed flask fitted with a pH electrode and stirrer. The pH was adjusted to 8.5 with aq. 0.1M-NH_3 and the thiol was determined (10μl portions) by the method of Ellman (1959). Acetic anhydride (4.5μl) was added with stirring and the pH was maintained at 8.5 by the dropwise addition of aq. 0.1M-NH_3. If any free thiol remained, a further amount of acetic anhydride (1.0μl) was added. After stirring for 2min at pH8.5, the pH was adjusted to 5 with 1M-acetic acid and the solution was freeze-dried. The acetyl-CoA was stored at −20°C in a desiccator. The purity was greater than 90% as judged by the thiol ester determination of Lipmann & Tuttle (1945).

Acetoacetyl-CoA was prepared by the method of Wieland & Rueff (1953).

Preparation of cytoplasmic β-oxoacyl-CoA thiolase. β-Oxoacyl-CoA thiolase was prepared in 10% yield from the livers of 15 Sprague–Dawley rats (Middleton, 1974). The enzyme was homogeneous as judged by polyacrylamide-gel electrophoresis and had a specific activity of 46.2–49.8units/mg using the assay method of Middleton (1973), although the peak fraction from the final column chromatography had a specific activity of 81.6units/mg. One unit of enzyme is defined as that required to consume 1μmol of acetoacetyl-CoA/min at 30°C.

Synthesis of acetoacetyl-CoA with thiolase. The incubation conditions employed for the optimal yield of acetoacetyl-CoA were as follows. A final volume of 1ml contained Tris/HCl buffer, pH8.8 (100μmol), $MgSO_4,7H_2O$ (300μmol), acetyl-CoA (70nmol) and thiolase (9.0 units; 4.45nmol; 195μg). The reaction was carried out in a spectrophotometer cell at 30°C and the formation of acetoacetyl-CoA was followed at 303nm. Experiments involving radioactive synthesis followed this method with only minor modifications to enzyme and substrate concentrations. Additions are shown in Table 1.

Preparation of acetyl–enzyme complex. The method of Gehring & Harris (1970) was modified as described below. Unlabelled acetyl–enzyme (see Expt. 2, Table 1) was prepared from 5.1 units (2.51nmol) of enzyme and 0.56μmol of acetyl-CoA at pH7. The acetyl–enzyme complex was separated from small molecules by gel filtration with Sephadex G-50 fine at 0°C. The disappearance of the reactive enzyme thiol (Ellman, 1959) indicated that the acetylation of the enzyme was complete.

[1-^{14}C]Acetyl–enzyme was prepared by reacting enzyme (4.45nmol) with [1-^{14}C]acetyl-CoA (22.1nmol). The yield of complex was 50–60% (see Expts. 3, 4 and 5 in Table 1).

Single-turnover experiments. Single-turnover experiments were carried out using a rapid mixing device with three syringe inputs, two of which allow enzyme and substrate (either ^{14}C or unlabelled) to interact for approximately 50ms. The third input allows a further addition of substrate (^{14}C or unlabelled) before final quenching of the reaction ~100ms later. In some experiments enzyme–substrate complex was used in the first syringe and additional substrate in the second leaving the third input for a rapid quench with 0.2M-NaOH. Details of quantities, ^{14}C label used and composition of experiments are given in Table 1.

In all experiments, the formation of acetoacetyl-CoA was promoted by the inclusion of $MgSO_4$ (0.3M final concentration), which chelates the enol form of acetoacetyl-CoA (Stern, 1956).

The randomization of label in acetoacetyl-CoA was minimized by utilization of methylmethanethiolsulphonate, which reacted with the liberated

CoA and prevented further reaction of the enzyme (Bloxham *et al.*, 1978).

Degradation of acetoacetyl-CoA to acetone semicarbazone and $BaCO_3$. The radioactivity in regiospecifically labelled acetoacetyl-CoA synthesized from [1-^{14}C]acetyl-CoA was determined at the end of the enzyme incubation as follows. The pH was adjusted to 13 with 5M-NaOH to terminate the enzyme reaction and to hydrolyse the thioester. After 10min at 37°C, carrier lithium acetoacetate (100mg) was added and the solution was centrifuged (1000*g*) for 5min to collect denatured enzyme. The supernatant was transferred into the left side of a two-arm 'soldier' and the pH was adjusted to 4 with 5M-HCl. Semicarbazide (200mg) and sodium acetate (200mg) were added to the solution. Into the right arm of the 'soldier' 7ml of saturated aqueous $Ba(OH)_2$ was added and the 'soldier' was stoppered. Decarboxylation was allowed to proceed for 16h at 37°C after which time the contents of the soldier were cooled to 0°C to complete the crystallization of acetone semicarbazone. Acetone semicarbazone and $BaCO_3$ were each isolated by filtration. Acetone semicarbazone was recrystallized from water to constant specific activity (m.p. 189°C).

Counting of radioactive samples. All determinations were made using a Phillips P4700 scintillation spectrometer programmed to give a quench correction to d.p.m. Samples were counted in 10ml of toluene/methanol (4:1, v/v) containing 5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6g/litre). $Ba^{14}CO_3$ precipitates were sonicated for 10min in the scintillation vial and the suspension was allowed to settle for 10min before counting radioactivity. The efficiency of the counting of $Ba^{14}CO_3$ was 73.9% as determined by conversion of a known quantity of $NaH^{14}CO_3$ into $Ba^{14}CO_3$.

Results and discussion

The mechanism by which β-oxoacyl thiolase from mammalian liver cytoplasm catalyses the formation of acetoacetyl-CoA from two molecules of acetyl-CoA is considered to follow an ordered Bi Bi reaction in which one of the acetyl-CoA molecules initially binds to the enzyme establishing an acyl–enzyme complex, with concomitant liberation of CoA. The acetyl–enzyme complex reacts with a second molecule of acetyl-CoA to yield the product acetoacetyl-CoA.

Acetyl-CoA has the unique property of acting either as a nucleophilic or as an electrophilic reagent. Two broad mechanisms are thus possible for the β-oxoacyl thiolase reaction. In mechanism (1) acetyl-CoA interacts with the enzyme with release of CoA and the establishment of an acyl–enzyme complex. Nucleophilic attack by the second acetyl-CoA molecule on the electrophilic thioester carbonyl group of the acetyl–enzyme complex liberates the product acetoacetyl-CoA. In this mechanism the initially bound acyl unit becomes incorporated into C-3 and C-4 of the product (Scheme 1).

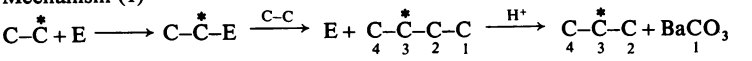
Equally feasible is the alternative mechanism (2) in which the initially bound acyl unit acts as a nucleophile, reacting with the carbonyl group of acetyl-CoA, the displaced CoA being used to cleave the acyl–enzyme bond to furnish acetoacetyl-CoA. In this latter mechanism, the initially bound acyl unit becomes incorporated into C-1 and C-2 of the product (Scheme 1).

By analogy with long-chain β-oxoacyl thiolases and from the study of exchange reactions (Gehring & Lynen, 1972) mechanism (1) is considered the most likely and has been favoured for several years.

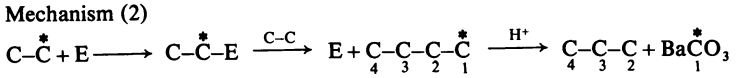
A knowledge of the order of binding of substrates to an enzyme is of paramount importance for the formulation of the reaction mechanism. In heterosubstrate reaction studies much information about the order of substrate binding can be obtained from steady-state kinetics (Cleland, 1967). However, in homopolymerization reactions of the β-oxoacyl-CoA thiolase type, where more than one molecule of the same substrate is involved, this approach is of little use. In this case the order of binding of the two identical substrate molecules can only be delineated by using a single-enzyme-turnover reaction in which one substrate molecule is distinguished from the other by isotopic labelling.

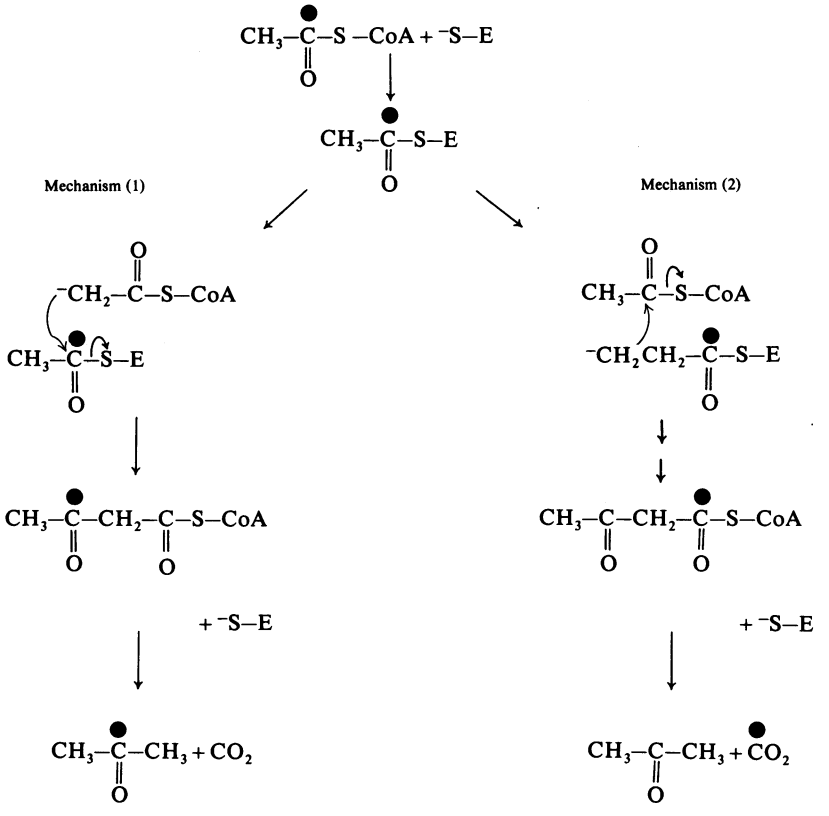
Accordingly, β-oxoacyl-CoA thiolase is mixed with a stoichiometric equivalent of [1-^{14}C]acetyl-CoA on anticipation that one of the two substrate binding sites will be preferentially occupied by the label. Subsequent addition of a large quantity of unlabelled acetyl-CoA to complete the turnover of the ^{14}C intermediate complex should thus result in the formation of acetoacetyl-CoA in which the majority of the label is located regiospecifically either at C-1 or at C-3 depending on which of the two acetyl-CoA binding sites was occupied initially by the labelled substrate.

Mechanism (1)



Mechanism (2)





Scheme 1. *Possible mechanisms for the β-oxoacyl-CoA thiolase reaction*
The black dot (●) enables the fate of the carbonyl carbon atom to be followed in both mechanisms.

Degradation of the acetoacetyl-CoA to determine the location of the label would thus provide direct evidence for the order of binding of the substrates to the enzyme and thus allow important mechanistic conclusions. This type of single-enzyme-turnover approach has been used successfully in this laboratory to elucidate mechanistic features of the enzymes 5-aminolaevulinic acid dehydratase (Jordan & Seehra, 1980*a*,*b*) and porphobilinogen deaminase (hydroxymethylbilane synthase) (Jordan & Seehra, 1979), both of which catalase homopolymerization reactions in tetrapyrrole biosynthesis.

Several difficulties associated with the study of the β-oxoacyl thiolase reactions were overcome as follows. First, the enzyme reaction normally favouring thiolysis ($K_{eq.}$ 5.8×10^{-5} at pH 8.5; Goldman, 1954) was encouraged in the direction of acetoacetyl-CoA synthesis by carrying out incubations in the presence of Mg^{2+}. This stabilizes the enol acetoacetyl-CoA as its magnesium chelate and effectively reduces the concentration of free acetoacetyl-CoA (Stern, 1956; Middleton, 1974). Secondly, the reaction was carried out in a rapid mixing device at 0°C such that the enzyme was in contact with the initial labelled substrate for 50ms, reducing the chance of both of the acetyl-CoA binding sites being sequentially occupied by label. Thirdly, after reaction with excess unlabelled acetyl-CoA, the reaction mixture was quenched into thiophilic reagents, which had the dual effect of removing CoASH and of inactivating the enzyme, thus reducing the reverse reaction and any attendant exchange.

The rapid-mixing device employed was fitted with three syringe inputs. This apparatus allows the enzyme and labelled substrate (in approximately stoichiometric quantities) to interact for ~50ms before the unlabelled substrate is added through the third input. The solution was then passed into methylmethanethiosulfonate and iodoacetamide at alkaline pH to terminate the reaction.

Thus, enzyme and [1-^{14}C]acetyl-CoA were initially mixed, excess unlabelled acetyl-CoA was added and after termination of the reaction the acetoacetyl-CoA was degraded to acetone and CO_2. The proportion of radioactivity in C-3, as acetone

semicarbazone, and C-1, as $BaCO_3$, could then be determined accurately. The ratio of label in C-3 and C-1 in this experiment (Expt. 1 in Table 1) was 68:32 showing that the molecule of acetyl-CoA initially bound to the enzyme became C-3 and C-4 in acetoacetyl-CoA. The converse experiment in which unlabelled acetyl-CoA was added initially to the enzyme, followed by [1-^{14}C]acetyl-CoA, gave, as expected, the converse distribution of label in acetoacetate with a C-3:C-1 ratio of 43:57 (Expt. 2 in Table 1). These results favour mechanism (1) discussed above.

To determine that the acyl moiety of the acetyl–enzyme complex (Gehring & Harris, 1970) was incorporated into positions C-3 and C-4 of acetoacetyl-CoA, [1-^{14}C]acetyl–enzyme was prepared (see the Experimental section), and subsequently allowed to react with excess unlabelled acetyl-CoA (Expts. 3 and 4). Degradation of the acetoacetyl-CoA showed that the C-3:C-1 ratio was 86:14 and 83:17 respectively, indicating that the reaction course using [1-^{14}C]acetyl–enzyme complex was the same as in the single-turnover experiments starting from [1-^{14}C]acetyl-CoA. If the reaction using [^{14}C]acetyl–enzyme complex was repeated (Expt. 5) but termination was delayed for 2 min the distribution of label was less marked and showed a C-3:C-1 ratio of 57:43, presumably due to equilibration with residual CoASH.

Expt. 6, in which excess [1-^{14}C]acetyl-CoA was added to the thiolase, yielded, as expected, an equal (50:50) distribution of radioactivity between C-3 and C-1 in the acetoacetyl-CoA. In control experiments, negligible radioactivity was incorporated into acetoacetyl-CoA (Expt. 7).

The data provide powerful direct evidence for the order of addition of the two substrate molecules in the β-oxoacyl-CoA thiolase reaction in which the initially bound two-carbon unit is incorporated into C-3 and C-4 of acetoacetyl-CoA (mechanism 1).

Table 1. *Enzyme-single-turnover experiments with β-oxoacyl-CoA thiolase*

All experiments were terminated with 0.2M-NaOH (300μl). In Expts. 1 and 2 the NaOH was in the termination vessel; in Expts. 3, 4 and 7 the NaOH was in syringe input C. In Expts. 5 and 6 2 min was allowed to elapse before termination. $MgSO_4,7H_2O$ (150μmol) and methylmethanethiosulphonate (MMTS; 5μmol) were present in all experiments. Quantities of enzyme and acetyl-CoA were as shown. All samples, after termination and the addition of carrier lithium acetoacetate (100mg), were processed as described in the Experimental section to give acetone semicarbazone and $BaCO_3$. The specific radioactivity of the [1-^{14}C]acetyl-CoA was 56.6 Ci/mol. Enzymic conversions of acetyl-CoA into acetoacetyl-CoA were typically approx. 24% (Expt. 6). In experiments in which [^{14}C]acetyl–enzyme was used (Expts. 3 and 4) approx. 50–60% of the label remained associated with the enzyme pellet after the experiment. This is attributed to an abortive enzyme $-N-COCH_3$ linkage formed by rearrangement of the enzyme $S-COCH_3$ moiety under the experimental conditions used (Holland *et al.*, 1973).

Expt.	Syringe input A	Syringe input B	Syringe input C	Sp. radioactivity (d.p.m./mmol) Acetone semicarbazone	Sp. radioactivity (d.p.m./mmol) $BaCO_3$	Ratio of specific activities in C-3 to C-1 of acetoacetic acid
1	Enzyme (4.45 nmol)	[^{14}C]Acetyl-CoA (22.1 nmol)	Acetyl-CoA (1.78μmol) Mg^{2+}, MMTS	5203	2450	68:32
2	Acetyl–enzyme (2.51 nmol)	[^{14}C]Acetyl-CoA (22.1 nmol)	Acetyl-CoA (1.78μmol) Mg^{2+}, MMTS	1636	2133	43:57
3	[^{14}C]Acetyl–enzyme (4.45 nmol)	Acetyl-CoA (1.78μmol) Mg^{2+}, MMTS	—	631	104	86:14
4	[^{14}C]Acetyl–enzyme (4.45 nmol)	Acetyl-CoA (11.8μmol) Mg^{2+}, MMTS	—	1100	229	83:17
5	[^{14}C]Acetyl–enzyme (4.45 nmol)	Acetyl-CoA (1.78μmol) Mg^{2+}, MMTS	—	1927	1430	57:43
6	Enzyme (4.45 nmol)	[^{14}C]Acetyl-CoA (70 nmol) Mg^{2+}, MMTS	—	78053	79387	50:50
7	Buffer	[^{14}C]Acetyl-CoA (22.1 nmol) Mg^{2+}, MMTS	—	36	0	—

The experiments also show conclusively that the acetyl moiety in the acyl–enzyme intermediate also gives rise to C-3 and C-4 of the acetoacetyl-CoA.

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