The Kinetic Mechanism of 3-Hydroxy-3-methylglutaryl-Coenzyme A Synthase from Baker's Yeast

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1. The effect of independent variation of both acetyl-CoA and acetoacetyl-CoA on the initial velocity at pH 8.0 and pH 8.9 gives results compatible with a sequential mechanism involving a modified enzyme tentatively identified as an acetyl-enzyme, resulting from the reaction with acetyl-CoA in the first step of a Ping Pong (Cleland, 1963*a*) reaction. 2. Acetoacetyl-CoA gives marked substrate inhibition that is competitive with acetyl-CoA. This suggests formation of a dead-end complex with the unacetylated enzyme and is in accord with the inhibition pattern given by 3-oxohexanoyl-CoA, an inactive analogue of acetoacetyl-CoA. 3. The inhibition pattern given by products of the reaction is compatible with the above mechanism. CoA gives mixed inhibition with respect to both substrates, whereas DL-3-hydroxy-3-methylglutaryl-CoA competes with acetyl-CoA but gives uncompetitive inhibition with respect to acetoacetyl-CoA. 4. 3-Hydroxy-3-methylglutaryl-CoA analogues lacking the 3-hydroxyl group are found to compete, like 3-hydroxy-3-methylglutaryl-CoA, with acetyl-CoA but have K_i values ninefold higher, indicating the importance of the 3-hydroxyl group in the interaction. 5. A comparison of inhibition by CoA and desulpho-CoA at pH 8.0 and pH 8.9 shows that at the higher pH value a kinetically significant reversal of the formation of acetyl-enzyme can occur. 6. Acetyl-CoA homologues do not act as substrates and compete only with acetyl-CoA. A study of the variation of K_i with acyl-chain length suggests the presence near the active centre of a hydrophobic region. 7. These results are discussed in terms of a kinetic mechanism in which there is only one CoA-binding site the specificity of which is altered by acetylation of the enzyme. 8. The rate of 3-hydroxy-3-methylglutaryl-CoA synthesis in yeast is calculated from the kinetic constants determined for purified 3-hydroxy-3-methylglutaryl-CoA synthase and from estimates of the physiological substrate concentrations. The rate of synthesis of 12 nmol of 3-hydroxy-3-methylglutaryl-CoA/min per g wet wt. of yeast is still greater than the rate of utilization in spite of the extremely low (calculated) acetoacetyl-CoA concentration (1.8 nM).

In the present paper the steady-state kinetics of the reaction catalysed by 3-hydroxy-3-methylglutaryl-CoA synthase have been investigated to gain evidence relevant to the hypothesis proposed by Stewart & Rudney (1966*b*) and supported by the inhibition studies of Middleton & Tubbs (1972) that an acetyl-enzyme is a normal intermediate in the reaction mechanism.

In their investigation of a purified preparation of this enzyme from baker's yeast, Stewart & Rudney (1966*a*) could not perform a thorough kinetic analysis owing to gross contamination with acetoacetyl-CoA thiolase. The design of a purification procedure yielding yeast 3-hydroxy-3-methylglutaryl-CoA synthase free from thiolase activity (Middleton & Tubbs, 1972) has enabled this study to be made. A preliminary account of part of this work has appeared (Middleton, 1967).

Materials and Methods

Materials

Enzymes and proteins. The following purified enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.: citrate synthase (EC 4.1.3.7, from pig heart) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35, from pig heart). Crystalline carnitine acetyltransferase (EC 2.3.1.7, from pigeon breast muscle) was a gift from Dr. J. F. A. Chase.

Acyl-CoA synthetase (EC 6.2.1.2) was a gift from Dr. D. J. Pearson.

3-Hydroxy-3-methylglutaryl-CoA-cleavage enzyme (EC 4.1.3.4, from ox liver) was prepared by the method of Middleton & Apps (1969).

3-Hydroxy-3-methylglutaryl-CoA synthase (EC

4.1.3.5, from baker's yeast) was purified to an average specific activity of 2 units/mg of protein by the method of Middleton & Tubbs (1972). The preparation was virtually free from acetoacetyl-CoA thiolase activity (0.004 unit/mg) and acetyl-CoA hydrolase activity (0.004 unit/mg) and had a very low hydrolase activity towards acetoacetyl-CoA (0.015 unit/mg).

Chemicals. ATP and CoA were obtained from Boehringer Corp. (London) Ltd. The following chemicals were obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.; 5,5'-dithiobis-(2-nitrobenzoic acid), 3-methylglutaric anhydride, 3,3'-dimethylglutaric anhydride and diketene. The latter was distilled before use. Glutaric acid was from British Drug Houses Ltd., Poole, Dorset, U.K., and the anhydride was prepared by the method of Hilz *et al.* (1958) and recrystallized from diethyl ether. Thioglycolic acid was obtained from British Drug Houses Ltd. 3-Oxohexanoyl ethyl ester was the Purum grade of Fluka A.G., Buchs, Switzerland.

Buffers. Tris (Trizma Base; Sigma Chemical Co., St. Louis, Mo., U.S.A.) was adjusted with HCl to the required pH.

Methods

Preparation and assay of substrates. Acetyl-CoA, propionyl-CoA, DL-3-hydroxy-3-methylglutaryl-CoA and desulpho-CoA were prepared and assayed as described by Middleton & Tubbs (1972). Acetoacetyl-CoA, prepared by the method of Wieland & Rueff (1953), contained up to 20% of a thiol ester with absorption maximum at 303 nm at pH 8.2, but which was not a substrate for 3-hydroxy-3-methylglutaryl-CoA synthase. Pure acetoacetyl-CoA was obtained as the lithium salt after chromatography of the impure preparation on DEAE-cellulose as described by Moffatt & Khorana (1961) for CoA. The impurity with an extinction at 303 nm was not retained by the column. This material appeared to be an artifact of the treatment of CoA with diketene, as there was no evidence of such impurity in the CoA or in acyl-CoA compounds produced by other methods.

3-Oxohexanoyl-CoA was prepared by thiol exchange from 3-oxohexanoyl thioglycollate and CoA (Vagelos & Alberts, 1960).

Butyryl-, glutaryl-, DL-3-methylglutaryl- and 3,3'-dimethylglutaryl-CoA were prepared by treating CoA with the appropriate acid anhydrides by the method of Simon & Shemin (1953).

Hexanoyl-, heptanoyl-, octanoyl-, nonanoyl- and decanoyl-CoA, prepared by the reaction of CoA with the mixed anhydride of the fatty acid concerned and ethyl chloroformate, were gifts from Dr. J. F. A. Chase.

3-Oxohexanoyl-CoA and acetoacetyl-CoA were assayed by the method of Decker (1963). Glutaryl-,

DL-3-methylglutaryl- and 3,3'-dimethylglutaryl-CoA were determined by their E_{260} by using the extinction coefficient for adenine nucleotides of $16.4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Stadtman, 1957).

CoA was determined by coupling to sorbate in the presence of ATP and acyl-CoA synthetase (Wakil & Hübscher, 1960).

Butyryl-, hexanoyl-, heptanoyl-, octanoyl-, nonanoyl- and decanoyl-CoA were determined by the method of Chase (1967).

The thiol content of acyl-CoA compounds was determined by the method of Ellman (1959) and was never more than 3% of the total thiol ester content.

Units of 3-hydroxy-3-methylglutaryl-CoA synthase activity. A unit is defined as the amount of this enzyme necessary to transform $1 \mu\text{mol}$ of substrate into product under the standard conditions of assay (Middleton & Tubbs, 1972).

Measurements of reaction rates. Initial-rate measurements were made at 303 nm in a Beckman DK-2 recording spectrophotometer fitted with a scale-expansion device (Dixon, 1967) and a temperature-controlled cell housing kept at 30°C. The assay medium comprised 100 mM-tris buffer, 20 mM-MgCl₂, $10 \mu\text{l}$ of 3-hydroxy-3-methylglutaryl-CoA synthase (5–20 μg of protein), substrates, products or analogues as required and water to a final volume of 2.0 ml. The reaction was started by the addition of enzyme to the otherwise complete system, which had been incubated at 30°C for 3 min. Over the pH range examined (pH 8.0–8.9) no detectable non-enzymic hydrolysis of acetyl-CoA occurred. It was found that Mg^{2+} increased the stability of acetoacetyl-CoA to non-enzymic hydrolysis and that in the presence of Mg^{2+} the stability increased with pH. Where a non-enzymic hydrolysis of acetoacetyl-CoA did occur the rate was subtracted from the initial reaction rate. As discussed in the preceding paper (Middleton & Tubbs, 1972), the enzymic hydrolysis of substrates was negligible compared with the full reaction rate.

Determination of apparent extinction coefficient of acetoacetyl-CoA. Stern (1956) has shown that the apparent extinction coefficient of acetoacetyl-CoA at 303 nm is increased by bivalent cations (especially Mg^{2+}) and by pH. The apparent extinction coefficient of purified acetoacetyl-CoA was determined under the assay conditions used here and was found to be $17.9 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (tris, pH 8.0) and $27.0 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (tris, pH 8.9).

Expression of results. Initial velocities are expressed as nmol of acetoacetyl-CoA transformed/min by using the appropriate extinction coefficient. All velocities are corrected to an enzyme concentration of $10 \mu\text{g}/\text{cuvette}$. Acetoacetyl-CoA concentrations were total concentrations and no attempt was made to allow for the various contributions by free keto or enol forms or their respective chelates with Mg^{2+} .

Where a DL-acyl-CoA was added the concentration refers to the total amount of both species present.

Results

Specificity for acyl group

3-Hydroxy-3-methylglutaryl-CoA synthase was found to be absolutely specific for acetyl-CoA and acetoacetyl-CoA in the reaction:

Neither propionyl- nor butyryl-CoA could replace acetyl-CoA and no reaction was observed when 3-oxohexanoyl-CoA was substituted for acetoacetyl-CoA.

Effect of substrate concentration on initial velocity

This was done for a wide range of acetyl- and acetoacetyl-CoA concentrations at pH 8.0 and 8.9 in tris buffer and the results are shown in Figs. 1 and 2. At pH 8.9 in Fig. 2(a) a series of parallel lines are shown, the apparent $V_{\max}$ and K_m for acetoacetyl-CoA being a function of the concentration of acetyl-CoA. For high concentrations of acetoacetyl-CoA, especially when the concentration of acetyl-CoA is low, the lines become sharply curved upwards, indicating high substrate inhibition by acetoacetyl-CoA, competitive with acetyl-CoA binding. At pH 8.0 this high substrate inhibition is very pronounced and the results in Fig. 1(a) are best expressed by a Dixon (1953) plot. In Fig. 1(b) the competitive nature of the inhibition with respect to acetyl-CoA is clearly illustrated.

These results, although complicated by marked substrate inhibition, suggest a kinetic mechanism in which the reaction proceeds through a covalently modified form of the enzyme and an ordered series of binary complexes but without the formation of kinetically significant amounts of ternary complex. Such a mechanism has been termed 'Ping Pong' by Cleland (1963a). From the inhibition studies in the preceding paper (Middleton & Tubbs, 1972) and from the studies by Stewart & Rudney (1966b) it would appear that the covalently modified intermediate is an acetyl-enzyme resulting from interaction with acetyl-CoA. The reaction would then occur in two stages:

where Ac is acetyl, Acac is acetoacetyl and HMG is 3-hydroxy-3-methylglutaryl. The CoASH released in reaction (1) should thus be derived from acetyl-CoA and this has been clearly shown by Ferguson & Rudney (1959).

Substrate inhibition by acetoacetyl-CoA that is competitive with acetyl-CoA would be explained by the formation of a dead-end complex between acetoacetyl-CoA and E, thus decreasing the binding

of acetyl-CoA and the subsequent formation of the acetyl-enzyme.

The initial-velocity equation compatible with these results is:

$$v = \frac{V}{1 + \frac{K_m^{s_1}}{[S_1]} \left(1 + \frac{[S_2]}{K_i^{s_2}} \right) + \frac{K_m^{s_2}}{[S_2]}} \quad (3)$$

where S_1 , the first substrate to bind to the enzyme in the ordered reaction sequence represents acetyl-CoA and S_2 represents acetoacetyl-CoA. $K_m^{s_1}$ is the concentration of acetyl-CoA giving a half-maximal velocity when acetoacetyl-CoA is saturating and $K_m^{s_2}$ is that concentration of acetoacetyl-CoA giving a half-maximal velocity when acetyl-CoA is saturating. The term $[S_2]/K_i^{s_2}$ represents inhibition by acetoacetyl-CoA where $K_i^{s_2}$ is the dissociation constant of an abortive complex between the free enzyme and acetoacetyl-CoA.

When

$$\frac{K_m^{s_1}}{[S_1]} \cdot \frac{[S_2]}{K_i^{s_2}} \gg \frac{K_m^{s_2}}{[S_2]} \quad (4)$$

eqn. (1) reduces to:

$$v = \frac{V}{1 + \frac{K_m^{s_1}}{[S_1]} \left(1 + \frac{[S_2]}{K_i^{s_2}} \right)} \quad (5)$$

and as Dixon (1953) has shown a plot of $1/v$ against $[S_2]$ at different values of $[S_1]$ gives a family of lines intersecting at an abscissa value of $-K_i^{s_2}$ and an ordinate value of $1/V$. This is shown in Fig. 1. The value of $K_m^{s_1}$ can be obtained from a secondary plot of the intercepts of Fig. 1 against $1/[S_1]$.

When the value of inequality (4) is reversed the

Fig. 1. Effect of different substrate concentrations on 3-hydroxy-3-methylglutaryl-CoA synthase activity at pH 8.0

(a) Reciprocal of the velocity plotted as a function of acetoacetyl-CoA concentration at different acetyl-CoA concentrations: $100 \mu\text{M}$ (Δ), $39.8 \mu\text{M}$ ($\circ$), $19.9 \mu\text{M}$ ($\triangle$), $14.9 \mu\text{M}$ ($\bullet$) and $9.95 \mu\text{M}$ ($\square$). (b) Double-reciprocal plot of velocity against acetyl-CoA concentration at different acetoacetyl-CoA concentrations: $25.5 \mu\text{M}$ (Δ), $17.8 \mu\text{M}$ ($\bullet$), $10.2 \mu\text{M}$ ($\square$) and $5.1 \mu\text{M}$ ($\circ$).

Fig. 2. Effect of different substrate concentrations on reciprocal plots of the velocity of the 3-hydroxy-3-methylglutaryl-CoA synthase-catalysed reaction at pH 8.9

(a) For acetoacetyl-CoA at different acetyl-CoA concentrations: $51 \mu\text{M}$ (Δ), $17.8 \mu\text{M}$ ($\bullet$), $10.2 \mu\text{M}$ ($\square$), $7.67 \mu\text{M}$ ($\triangle$) and $5.1 \mu\text{M}$ ($\circ$); (b) for acetyl-CoA at different acetoacetyl-CoA concentrations: $19.1 \mu\text{M}$ (Δ), $9.55 \mu\text{M}$ ($\bullet$), $3.34 \mu\text{M}$ ($\circ$), $1.91 \mu\text{M}$ ($\square$), $1.43 \mu\text{M}$ ($\triangle$).

Fig. 2.

initial velocity equation reverts to the usual hyperbolic form:

$$v = \frac{V}{1 + \frac{K_m^{S_1}}{[S_1]} + \frac{K_m^{S_2}}{[S_2]}} \quad (6)$$

The kinetic results at pH 8.9 (when the ratio of acetyl-CoA to acetoacetyl-CoA is high) conforms to this equation. The values of V , $K_m^{S_1}$ and $K_m^{S_2}$ can be obtained from secondary plots.

In the general case of adherence to eqn. (3) all the kinetic parameters can be obtained from the slopes and intercepts of a plot of $1/v$ against $1/[S_1]$. This is a series of straight lines at all values of $[S_1]$ and $[S_2]$ (see Figs. 1 and 2) with:

$$\text{slopes} = K_m^{S_1} \left(1 + \frac{[S_2]}{K_i^{S_2}} \right) \quad (7)$$

$$\text{and intercepts} = \frac{1}{V} \left(1 + \frac{K_m^{S_2}}{[S_2]} \right) \quad (8)$$

Secondary plots of eqn. (7) against $[S_2]$ and eqn. (8) against $1/S_2$ yield values for V , $K_m^{S_1}$, $K_m^{S_2}$ and $K_i^{S_2}$. Secondary plots for the evaluation of these parameters from the results in Fig. 2 are shown in Fig. 3. The values of V , $K_m^{S_1}$, $K_m^{S_2}$ and $K_i^{S_2}$ obtained from these studies are shown in Table 1. The value of the K_m for acetoacetyl-CoA at saturating values of acetyl-CoA ($K_m^{S_2}$) at pH 8.0 is extremely low and can only be estimated from eqn. (4).

Inhibition by products of the reaction

The patterns of product inhibition can often be of great help in determining reaction pathways (Cleland, 1963a). The effect of including fixed concentrations of CoA and DL-3-hydroxy-3-methylglutaryl-CoA on the initial velocity for different acetyl- and acetoacetyl-CoA concentrations was investigated at pH 8.0 and the results are presented in Figs. 4 and 5 respectively. The results are summarized in Table 2. The inhibition constants (K_i) are calculated by the method of Dixon (1953). The K_i for DL-3-hydroxy-3-methylglutaryl-CoA is expressed in terms of the total 3-hydroxy-3-methylglutaryl-CoA concentration, as the effects of the individual isomers have not been studied.

Fig. 5 shows that inhibition by DL-3-hydroxy-3-methylglutaryl-CoA is competitive with respect to acetyl-CoA and uncompetitive with respect to acetoacetyl-CoA. This type of inhibition is expected from a Ping Pong mechanism by a product that can only form one kinetically significant complex with one enzyme form. According to the proposed scheme (eqns. 1 and 2) it is reasonable to suppose that 3-hydroxy-3-methylglutaryl-CoA should bind to the

Fig. 3. Secondary plots of slopes and intercepts from the results in Fig. 2(b)

(a) Intercepts plotted as a function of reciprocal acetoacetyl-CoA concentration; (b) slopes plotted as a function of acetoacetyl-CoA concentration.

unacetylated enzyme and hence that it should compete with acetyl-CoA.

Inhibition by CoA, the other product, was found to be mixed with respect to each substrate. This pattern of inhibition indicates binding of CoA to both forms of the enzyme thus interfering with binding by both acetyl- and acetoacetyl-CoA substrates. The proposed reaction pathway that is consistent with these results is shown in Scheme 1. It is further proposed that there is only one binding site for the CoA moiety and that the specificity of the enzyme for the acyl portion of the substrate is altered by the acetylation that the enzyme undergoes in the first half of the reaction. Thus the unacetylated form of the enzyme will bind acetyl-CoA or 3-hydroxy-3-methylglutaryl-CoA, as predicted by

Table 1. *Kinetic constants for the reaction between acetyl-CoA and acetoacetyl-CoA at pH8.0 and pH8.9*

Constants were obtained from secondary plots of the results in Figs. 1 and 2 as described in the text.

pH	$V_{\max.}$ ($\mu\text{mol/min per mg}$)	K_m^{AcCoA} (μM)	K_m^{AcacCoA} (μM)	K_i^{AcacCoA} (μM)
8.0	3.0	14.0	$\leq 0.4^*$	8.0
8.9	3.0	18.0	3.2	20.0

* Estimated from eqn. (4).

Fig. 4. *Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by CoA at pH8.0*

(a) With respect to acetoacetyl-CoA at constant acetyl-CoA concentration ($100\mu\text{M}$); (b) with respect to acetyl-CoA at constant acetoacetyl-CoA concentrations ($4.4\mu\text{M}$). CoA concentrations: 0 (Δ), $55\mu\text{M}$ ($\circ$), $110\mu\text{M}$ ($\square$), $275\mu\text{M}$ ($\bullet$) and $550\mu\text{M}$ ($\blacksquare$).

eqns. (1) and (2), but the kinetic results also indicate ability to bind CoA and acetoacetyl-CoA in a dead-end complex. The kinetic results also suggest that binding to the acetylated form of the enzyme is restricted to acetoacetyl-CoA and CoA, the compounds that are involved in further chemical reaction with this species. The further investigations described

below were done to test this proposed reaction pathway.

Reversibility of the reaction

Although Scheme 1 shows the overall reaction to be reversible neither Ferguson & Rudney (1959) nor I have found any evidence of this. Over the pH range 8–9.25 there was no spectrophotometric evidence of acetoacetyl-CoA production from an assay system containing 1 unit of 3-hydroxy-3-methylglutaryl-CoA synthase, $0.2\mu\text{mol}$ of CoA and $0.44\mu\text{mol}$ of DL-3-hydroxy-3-methylglutaryl-CoA. Thus one or both of the partial reactions (eqns. 1 and 2) must be essentially irreversible. The product-inhibition results give some information on this subject. Thus, from Cleland's (1963b) rules the reversibility of acetyl-enzyme formation (eqn. 1) as shown in Scheme 1 predicts that inhibition by CoA, as well as being mixed towards both substrates, should be a non-linear function of the CoA concentration thus giving a concave-shaped Dixon plot. Fig. 6(a) shows that no departure from linearity is evident at pH 8.0, thus indicating that reversal of acetyl-enzyme formation is not kinetically significant under those conditions. At pH 8.9, however, the Dixon (1953) plot shows significant departure from linearity for inhibition by CoA, whereas at the same pH value inhibition by desulpho-CoA, a chemically inert CoA analogue, gives a linear plot (Fig. 6b). This clearly suggests that at pH 8.9 (but not at pH 8) the binding of CoA to the acetyl-enzyme results in a kinetically significant reversal of the acetyl-enzyme formation (eqn. 1). Desulpho-CoA can bind to the acetyl-enzyme, but, because it lacks the necessary thiol group, cannot react further.

The possibility of reversal of eqn. (2), the discharge of acetyl-enzyme by 3-hydroxy-3-methylglutaryl-CoA formation, can be tested for similarity. Cleland's (1963b) rules would predict from Scheme 1 that, should reversal be kinetically significant, inhibition by 3-hydroxy-3-methylglutaryl-CoA would remain competitive with respect to acetyl-CoA but would be mixed with respect to acetoacetyl-CoA. Fig. 5 shows that at pH 8 there is no indication of mixed inhibition, which argues against any significant reversal

Fig. 5. Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by DL-3-hydroxy-3-methylglutaryl-CoA at pH 8.0

(a) With respect to acetoacetyl-CoA at constant acetyl-CoA concentration (143 μM); (b) with respect to acetyl-CoA at constant acetoacetyl-CoA concentration (4.5 μM). DL-3-Hydroxy-3-methylglutaryl-CoA concentrations: 0 (Δ), 27.5 μM ($\bullet$), 55 μM ($\square$), 110 μM ($\circ$) and 220 μM ($\blacktriangle$).

of this study), the steps $E \cdot AcCoA \rightarrow E-Ac \cdot CoA$ and $E-Ac \cdot AcacCoA \rightarrow E \cdot HMG-CoA$ (Scheme 1) can be regarded as irreversible. This means that the K_i values given in Table 2 represent true dissociation constants for the respective enzyme-product complexes.

Inhibition by derivatives of CoA

Inhibition by analogues of 3-hydroxy-3-methylglutaryl-CoA. The following analogues of 3-hydroxy-3-methylglutaryl-CoA were synthesized and tested as reversible inhibitors at pH 8.0: glutaryl-CoA, DL-3-methylglutaryl-CoA and 3,3'-dimethylglutaryl-CoA. As none of these compounds contains a 3-hydroxyl group there is no possibility of enzyme-catalysed scission of the carbon skeleton as in the reversal of eqn. (2) with 3-hydroxy-3-methylglutaryl-CoA. All these compounds were found to be reversible inhibitors and all gave inhibition patterns qualitatively identical with that described for DL-3-hydroxy-3-methylglutaryl-CoA. The results for glutaryl-CoA are shown in Fig. 7 and are typical of the group. The K_i values for the members of the group are given in Table 3.

Inhibition by desulpho-CoA. Studies of the reversible inhibition by this unreactive CoA analogue enabled the proposals in Scheme 1 to be checked. Neither the inhibition patterns nor the calculated K_i values for desulpho-CoA can be complicated by the enzyme-catalysed chemical changes that could occur on binding of CoA to the active centre of the enzyme. The pattern of reversible inhibition by desulpho-CoA at pH 8.0 is shown in Fig. 8 and the K_i values calculated from these results are given in Table 3.

As with CoA, inhibition by desulpho-CoA was mixed with respect to both substrates. The close similarity shown between the K_i values for inhibition by desulpho-CoA and CoA verifies the suggestion that under these assay conditions reversal of acetyl-enzyme formation is not kinetically significant and that the K_i values calculated for CoA are true dissociation constants for the complexes $E \cdot CoA$ and $E-Ac \cdot CoA$.

Inhibition by homologues of acetyl-CoA. Reversible inhibition by a series of acyl-CoA thiol esters was studied at pH 8.0 under the usual assay conditions. The pattern of inhibition of all the compounds investigated was identical; i.e. competitive with respect to acetyl-CoA and uncompetitive with respect to acetoacetyl-CoA. Figs. 9 and 10 show results for inhibition by butyryl- and nonanoyl-CoA respectively. These are typical of the results obtained for the other acyl-CoA compounds. The K_i values calculated by the method of Dixon (1953) are listed in Table 4. The K_i values decrease with increasing carbon chain length from hexanoyl-CoA upwards. Studies on compounds of longer chain length than

of 3-hydroxy-3-methylglutaryl-CoA formation under these conditions.

Thus at pH 8 (the value used for the greater part

Table 2. *Reversible inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by products at pH8.0*

K_i values were obtained by the method of Dixon (1953) from Figs. 4 and 5.

Product CoA	Acetyl-CoA		Acetoacetyl-CoA	
	Nature of inhibition	K_i (μM)	Nature of inhibition	K_i (μM)
DL-3-Hydroxy-3-methylglutaryl-CoA	Mixed	38	Mixed	60
	Competitive	13	Uncompetitive	—

Scheme 1. *Proposed reaction pathway of 3-hydroxy-3-methylglutaryl-CoA synthesis from acetyl-CoA and acetoacetyl-CoA catalysed by yeast 3-hydroxy-3-methylglutaryl-CoA synthase*

Abbreviations: AcCoA, acetyl-CoA; AcacCoA, acetoacetyl-CoA; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; E, free enzyme; E-Ac, acetyl-enzyme.

decanoyl-CoA were prevented by the formation of insoluble complexes with the Mg^{2+} present in the assay system. There was no indication that the inhibition observed was other than reversible. Thus preincubation with $10\mu\text{M}$ -palmitoyl-CoA for up to 30min at 30°C and pH8.0 gave no increase in inhibition with time.

Inhibition by 3-oxohexanoyl-CoA. 3-Oxohexanoyl-CoA is an unreactive analogue of acetoacetyl-CoA and study of the patterns of reversible inhibition by this compound were done to test the proposal (Scheme 1) that acetoacetyl-CoA can bind to both forms of the enzyme.

The study was done at pH8.0 and the results are presented in Fig. 11. Like CoA and desulpho-CoA but unlike other acyl-CoA derivatives, 3-oxohexanoyl-CoA showed mixed inhibition towards both acetyl- and acetoacetyl-CoA. The K_i values are given in Table 4.

Discussion

The general equation for the initial velocity of an ordered two-substrate reaction (Alberty, 1953):

$$v = \frac{V_{\max.}}{1 + \frac{K_m^{S_1}}{[S_1]} + \frac{K_m^{S_2}}{[S_2]} + \frac{K^{S_1S_2}}{[S_1S_2]}} \quad (9)$$

Fig. 6. *Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by CoA and desulpho-CoA*

The effect of concentrations of CoA at fixed substrate concentrations was investigated. The acetyl-CoA concentration was $14.7\mu\text{M}$ and the acetoacetyl-CoA concentration was $3\mu\text{M}$. (a) Plot of reciprocal velocity as a function of CoA concentration at pH 8.0; (b) plot of reciprocal velocity as a function of CoA (Δ) and desulpho-CoA ($\blacktriangle$) concentrations at pH8.9.

includes a binary term $K^{S_1S_2}/[S_1S_2]$ that is absent if the mechanism is of the Ping Pong type. This absence yields the familiar parallel lines obtained when the initial velocities resulting from the independent

variation of both substrates are plotted by the method of Lineweaver & Burk (1934). This is seen for 3-hydroxy-3-methylglutaryl-CoA synthase in Fig. 2(a),

Fig. 7. Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by glutaryl-CoA at pH8.0

(a) With respect to acetoacetyl-CoA at a constant acetyl-CoA concentration ($90\mu\text{M}$); (b) with respect to acetyl-CoA at a constant acetoacetyl-CoA concentration ($4.5\mu\text{M}$). Glutaryl-CoA concentrations: $0\ (\Delta)$, $119\mu\text{M}\ (\bullet)$, $288\mu\text{M}\ (\square)$, $357\mu\text{M}\ (\circ)$ and $714\mu\text{M}\ (\blacksquare)$.

but in Fig. 1, under the conditions used for the greater part of this study, this pattern is lost because of the severity of the substrate inhibition by acetoacetyl-CoA. If, under these conditions, the initial velocity is measured at different concentrations of a

Fig. 8. Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by desulpho-CoA at pH 8.0

(a) With respect to acetoacetyl-CoA at a constant acetyl-CoA concentration ($80\mu\text{M}$); (b) with respect to acetyl-CoA at a constant acetoacetyl-CoA concentration ($4.4\mu\text{M}$). Desulpho-CoA concentrations: $0\ (\Delta)$, $40\mu\text{M}\ (\circ)$, $80\mu\text{M}\ (\square)$, $250\mu\text{M}\ (\bullet)$ and $550\mu\text{M}\ (\blacksquare)$.

Table 3. Reversible inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by product analogues at pH8.0

K_i values were obtained by the method of Dixon (1953) from Figs. 7 and 8.

Inhibitor	Acetyl-CoA		Acetoacetyl-CoA	
	Nature of inhibition	$K_i\ (\mu\text{M})$	Nature of inhibition	$K_i\ (\mu\text{M})$
Desulpho-CoA	Mixed	30	Mixed	70
Glutaryl-CoA	Competitive	110	Uncompetitive	—
DL-3-Methylglutaryl-CoA	Competitive	100	Uncompetitive	—
3,3'-Dimethylglutaryl-CoA	Competitive	120	Uncompetitive	—

Fig. 9. Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by butyryl-CoA at pH 8.0

(a) With respect to acetoacetyl-CoA at a constant acetyl-CoA concentration ($90 \mu\text{M}$); (b) with respect to acetyl-CoA at a constant acetoacetyl-CoA concentration ($4.5 \mu\text{M}$). Butyryl-CoA concentrations: 0 (Δ), $31 \mu\text{M}$ ($\bullet$), $62.5 \mu\text{M}$ ($\square$), $93.8 \mu\text{M}$ ($\circ$), $188 \mu\text{M}$ ($\blacktriangle$) and $376 \mu\text{M}$ ($\blacksquare$).

Fig. 10. Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by nonanoyl-CoA at pH 8.0

(a) With respect to acetoacetyl-CoA at a constant acetyl-CoA concentration ($105 \mu\text{M}$); (b) with respect to acetyl-CoA at a constant acetoacetyl-CoA concentration ($4.5 \mu\text{M}$). Nonanoyl-CoA concentrations: 0 (Δ), $14.9 \mu\text{M}$ ($\square$), $29.8 \mu\text{M}$ ($\circ$), $74.5 \mu\text{M}$ ($\blacktriangle$) and $149 \mu\text{M}$ ($\blacksquare$).

Table 4. Reversible inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by substrate analogues at pH 8.0

K_i values were obtained by the method of Dixon (1953) from Figs. 9 and 10.

Acyl-CoA	Acetyl-CoA		Acetoacetyl-CoA	
	Nature of inhibition	K_i (μM)	Nature of inhibition	K_i (μM)
3-Oxohexanoyl	Mixed	7.1	Mixed	14.0
Propionyl	Competitive	5.0	Uncompetitive	—
Butyryl	Competitive	33.0	Uncompetitive	—
Hexanoyl	Competitive	22.0	Uncompetitive	—
Heptanoyl	Competitive	12.3	Uncompetitive	—
Octanoyl	Competitive	8.1	Uncompetitive	—
Nonanoyl	Competitive	4.0	Uncompetitive	—
Decanoyl	Competitive	2.7	Uncompetitive	—

Fig. 11. Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by 3-oxohexanoyl-CoA at pH 8.0

(a) With respect to acetoacetyl-CoA at a constant acetyl-CoA concentration ($90\mu\text{M}$); (b) with respect to acetyl-CoA at a constant acetoacetyl-CoA ($4.5\mu\text{M}$). 3-Oxohexanoyl-CoA concentrations: 0 (Δ), $7.25\mu\text{M}$ ($\circ$), $14.5\mu\text{M}$ ($\blacksquare$), $29\mu\text{M}$ ($\square$).

Fig. 12. Effect of different substrate concentrations on 3-hydroxy-3-methylglutaryl-CoA synthase activity at pH 8.0

The reciprocal of the velocity is plotted as a function of the concentration of a mixture of acetyl-CoA and acetoacetyl-CoA in the ratio 1.95:1.

reactive analogues, 3-oxohexanoyl-CoA and desulpho-CoA respectively, give rise to mixed inhibition with respect to both substrates, a result that can only be explained by the ability of those analogues to form dead-end complexes with enzyme and acetyl-enzyme. It is, however, noteworthy that under the conditions of this study acetyl-CoA and its homologues and 3-hydroxy-3-methylglutaryl-CoA and its analogues are only able to combine with the unacetylated enzyme form. These acyl-CoA compounds are thus competitive inhibitors with respect to acetyl-CoA but uncompetitive with respect to acetoacetyl-CoA.

This behaviour completely justifies the hypothesis advanced in the Results section that the active centre of the enzyme contains one CoA-moiety-binding site, but that the specificity for the acyl portion of the substrate or product be altered by acetylation of the enzyme. Acetylated enzyme can bind only free CoA and 3-oxoacyl-CoA compounds, thus showing a far greater specificity than the unacetylated form. As it seems unlikely that mere acetylation could change the specificity so drastically a conformational change caused by acetylation may explain this behaviour.

Inhibition by acyl-CoA compounds that compete with acetyl-CoA gives information about the active site in the unacetylated enzyme. From hexanoyl-CoA the dissociation constant of the enzyme-acyl-CoA complex (K_i) decreases with increasing hydrocarbon chain length of the acyl portion (Table 4). In Fig. 13 the pK_i ($pK_i = -\log K_i$) is plotted against the chain length of the acyl-CoA. Butyryl-CoA has the same K_i value as CoA and desulpho-CoA but after hexanoyl-CoA there is a linear increase in pK_i with

fixed-ratio mixture of the two substrates, then according to eqn. (3), which purports to describe the mechanism of 3-hydroxy-3-methylglutaryl-CoA synthase, a plot of reciprocal velocity against the reciprocal of the mixed-substrate concentration should yield a straight line. If the mechanism is not Ping Pong then according to eqn. (9) the result should be a curve of increasing slope. This test was done at pH 8.0 for a fixed acetyl-CoA/acetoacetyl-CoA ratio of 1.95 over a tenfold concentration range and the result is shown in Fig. 12. The straight line shows that even under conditions of high acetoacetyl-CoA inhibition the kinetic mechanism is basically Ping Pong and that eqn. (3) is justified.

3-Hydroxy-3-methylglutaryl-CoA synthase is peculiar in that all substrates and products contain the CoA moiety. It is therefore not surprising to find that acetoacetyl-CoA and CoA can form dead-end complexes with the free enzyme as well as forming productive complexes with the acetyl-enzyme. This behaviour is confirmed by the finding that their non-

Fig. 13. Variation of pK_i ($-\log K_i$) for a number of acyl-CoA inhibitors of 3-hydroxy-3-methylglutaryl-CoA synthase with the number of carbon atoms in the side-chain acyl group

acyl chain length, showing an equal increment in pK_i for each additional $-\text{CH}_2-$ group equivalent to a ΔG° of binding of -1.2 kJ/mol (-0.30 kcal/mol) ($\Delta G^\circ = -RT \ln K_i$, where K_i is a true dissociation constant of the enzyme-inhibitor complex). It is therefore suggested that yeast 3-hydroxy-3-methylglutaryl-CoA synthase contains a hydrophobic region near the active centre with which an acyl-CoA hydrocarbon chain may interact, thus enhancing binding. Further, it is suggested that acyl-CoA hydrocarbon chains of less than six carbon atoms are not able to interact with this region and have the same affinity for the active site as unacylated CoA. The anomalously low value of the K_i obtained with propionyl-CoA (Prop-CoA) can be explained by the assumption that in this case further reaction can occur after initial binding to the enzyme:

Propionyl-CoA is not a substrate for the enzyme, and as inhibition by propionyl-CoA is not irreversible in the absence of added CoA, the proposed intermediate E-Prop-CoA should not rapidly dissociate to a propionyl-enzyme and free CoA. If the above reaction sequence could occur the apparent K_i for

propionyl-CoA would be equal to $\frac{k_{-1}k_{-2}}{k_{+1}(k_{-2}+k_{+2})}$ and can therefore be less than the dissociation constant of the E-Prop-CoA complex, k_{-1}/k_{+1} .

There is also evidence for a powerful hydrophilic interaction between a hydroxyl or oxo group on the third carbon atom of the acyl chain and the active centre of the unacetylated enzyme. Thus the K_i values of glutaryl-CoA and DL-3-methylglutaryl-CoA are almost ninefold greater than that for DL-3-hydroxy-3-methylglutaryl-CoA (assuming that the K_i for the latter is indeed a true dissociation constant). Similarly, comparison of the K_i values for acetoacetyl-CoA and 3-oxohexanoyl-CoA binding to the unacetylated enzyme with those for butyryl-CoA and hexanoyl-CoA respectively shows a three- to four-fold enhancement of binding by inclusion of the 3-oxo group.

In yeast the proposed role of 3-hydroxy-3-methylglutaryl-CoA synthase is to provide the precursor for synthesis of isoprenes and steroids (Rudney, 1959). Acetoacetyl-CoA must be provided from acetyl-CoA by acetoacetyl-CoA thiolase working in the synthetic direction and it has been shown (Middleton & Apps, 1969) that both this thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase are located in the cytoplasmic fraction of the yeast cell.

Owing to the unfavourable equilibrium of the thiolase reaction in the synthetic direction, it has been argued that in liver during mitochondrial ketone-body synthesis the concentration of acetoacetyl-CoA is unlikely to rise above $0.01 \mu\text{M}$ (Greville & Tubbs, 1968). These authors have therefore suggested that in liver mitochondria thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase may be part of a multienzyme complex and the substrate for 3-hydroxy-3-methylglutaryl-CoA synthase may be acetoacetyl-CoA bound to thiolase. There could thus be a high local concentration of acetoacetyl-CoA in the vicinity of 3-hydroxy-3-methylglutaryl-CoA synthase, making possible the observed (Krebs *et al.*, 1969) rapid rates of ketone-body synthesis.

These suggestions may also be applicable to steroid biosynthesis in yeast, and Stewart & Rudney (1966a) have proposed the existence of a complex between yeast thiolase and 3-hydroxy-3-methyl-

glutaryl-CoA synthase. If it exists this complex must be extremely labile, as these enzymes can be chromatographed on Sephadex G-200 without any sign of interaction (Middleton & Tubbs, 1972). It is now possible, by using the kinetic parameters determined above, to calculate whether the rate of 3-hydroxy-3-

methylglutaryl-CoA formation in the yeast cell at the prevailing low concentration of acetoacetyl-CoA would be sufficient to provide precursor material for steroid biosynthesis. The following assumptions are made.

(i) Acetoacetyl-CoA thiolase is sufficiently active to bring acetoacetyl-CoA and CoA into equilibrium with acetyl-CoA in yeast cytoplasm, and the concentration of cytoplasmic acetoacetyl-CoA is therefore governed by the relationship (Greville & Tubbs, 1968):

$$[\text{Acetoacetyl-CoA}] = [\text{acetyl-CoA}]^2 / [\text{CoA}] \times 5 \times 10^{-5}$$

(ii) The concentrations of cytoplasmic acetyl-CoA and CoA in yeast are comparable with those calculated by Williamson (1969) for rat liver cytoplasm during perfusion with lactate.

(iii) The kinetic mechanism for 3-hydroxy-3-methylglutaryl-CoA synthase, proposed in the present paper, applies *in vivo* and that the K_m for acetoacetyl-CoA at saturating acetyl-CoA concentrations is $0.1 \mu\text{M}$ (calculated to be $\ll 0.4 \mu\text{M}$ from eqn. 4). The other kinetic constants are taken from Table 1 and K_i values for CoA inhibition are from Table 2.

With the aid of these assumptions the estimated concentrations of the relevant CoA compounds in yeast cytoplasm are: acetyl-CoA, $60 \mu\text{M}$; CoA, $100 \mu\text{M}$; acetoacetyl-CoA, 1.8 nM . The equation governing 3-hydroxy-3-methylglutaryl-CoA synthesis under these conditions is:

$$v = \frac{V}{1 + \frac{K_m^{\text{AcCoA}}}{[\text{AcCoA}]} \left(1 + \frac{[\text{CoA}]}{K_i} \right) + \frac{K_m^{\text{AcacCoA}}}{[\text{AcacCoA}]} \left(1 + \frac{[\text{CoA}]}{K_{ii}} \right)}$$

$$K_m^{\text{AcCoA}} = 14 \mu\text{M}, K_i = 38 \mu\text{M};$$

$$K_m^{\text{AcacCoA}} = 0.1 \mu\text{M} \text{ (estimated)}, K_{ii} = 60 \mu\text{M};$$

from which the calculated velocity of 3-hydroxy-3-methylglutaryl-CoA synthesis is $V/170$. The value of V for fresh baker's yeast is $2 \mu\text{mol/min per g}$ fresh weight of yeast (B. Middleton, unpublished work). The expected rate of 3-hydroxy-3-methylglutaryl-CoA synthesis under the above conditions is therefore $12 \text{ nmol/min per g}$ fresh weight of yeast. This rate is several times the observed rate of 3-hydroxy-3-methylglutaryl-CoA removal catalysed by yeast 3-hydroxy-3-methylglutaryl-CoA reductase. Knappe *et al.* (1959) found the activity of 3-hydroxy-3-methylglutaryl-CoA reductase in baker's yeast to be $3.4 \text{ nmol/min per g}$ fresh weight. In liver this enzyme is known to be rate-limiting for the whole pathway of steroid biosynthesis (Siperstein & Fagan, 1966). If one assumes that this also applies to yeast it is

clear that the physiological rate of 3-hydroxy-3-methylglutaryl-CoA synthesis, even at the extremely low concentrations of acetoacetyl-CoA that must be present, is more than adequate to account for the requirements of steroid synthesis.

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