Biochem. J. (1985) **227**, 601–607
Printed in Great Britain

3-Hydroxy-3-methylglutaryl-coenzyme A synthase from ox liver

Properties of its acetyl derivative

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(*Received 30 October 1984/Accepted 20 December 1984*)

Ox liver mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) reacts with acetyl-CoA to form a complex in which the acetyl group is covalently bound to the enzyme. This acetyl group can be removed by addition of acetoacetyl-CoA or CoA. The extent of acetylation and release of CoA were found to be highly temperature-dependent. At temperatures above 20°C, a maximum value of 0.85 mol of acetyl group bound/mol of enzyme dimer was observed. Below this temperature the extent of rapid acetylation was significantly lowered. Binding stoichiometries close to 1 mol/mol of enzyme dimer were also observed when the 3-hydroxy-3-methylglutaryl-CoA synthase activity was titrated with methyl methanethiosulphonate or bromoacetyl-CoA. This is taken as evidence for a 'half-of-the-sites' reaction mechanism for the formation of 3-hydroxy-3-methylglutaryl-CoA by 3-hydroxy-3-methylglutaryl-CoA synthase. The $K_{eq.}$ for the acetylation was about 10. Isolated acetyl-enzyme is stable for many hours at 0°C and pH 7, but is hydrolysed at 30°C with a half-life of 7 min. This hydrolysis is stimulated by acetyl-CoA and slightly by succinyl-CoA, but not by desulpho-CoA. The site of acetylation has been identified as the thiol group of a reactive cysteine residue by affinity-labelling with the substrate analogue bromo[1-^{14}C]acetyl-CoA.

HMG-CoA synthase (EC 4.1.3.5) catalyses the formation of HMG-CoA from acetyl-CoA and acetoacetyl-CoA.

Kinetic and chemical experiments on HMG-CoA synthases isolated from yeast (Middleton, 1972; Middleton & Tubbs, 1972, 1974) and chicken liver (Miziorko *et al.*, 1975, 1976; Miziorko & Lane, 1977) have established that the enzymic formation of HMG-CoA proceeds via a Bi Bi Ping Pong kinetic mechanism, in which the enzyme reacts with acetyl-CoA to form a covalent acetyl-enzyme intermediate with the loss of CoA. The kinetic properties of the ox liver HMG-CoA synthase have also been examined, and these are compatible with such a mechanism (Page & Tubbs, 1978; Lowe & Tubbs, 1985). The present paper describes the formation of the acetyl derivative of the enzyme from ox liver and some of its properties.

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; E, enzyme; Ac-CoA, acetyl-CoA.

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Experimental

Materials

CoA and acetoacetyl-CoA were obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.). Acetyl-CoA was prepared by treating CoA with acetic anhydride (Simon & Shemin, 1953). Succinyl-CoA was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Desulpho-CoA was prepared by the action of Raney nickel on CoA (Chase *et al.*, 1966) and purified by passage through a Dowex 50W-X8 resin (BDH Chemicals, Poole, Dorset, U.K.). Bromoacetyl-CoA and bromo[1-^{14}C]acetyl-CoA (7.3 Ci/mol) were prepared as described by Lowe & Tubbs (1983). [1-^{14}C]Acetyl-CoA (4 or 5.55 Ci/mol) was obtained from Amersham International (Amersham, Bucks., U.K.).

Methyl methanethiosulphonate, 3-bromopyruvic acid, *N*-ethylmaleimide, Pronase (from *Streptomyces griseus*; Type XIV) and pepsin were obtained from Sigma, and trypsin (treated with tosylphenylalanylchloromethane) was obtained from Worthington Chemicals (Freehold, NJ, U.S.A.).

HMG-CoA synthase from ox liver mitochondria was purified and assayed as described by Lowe & Tubbs (1985). The final specific activity was between 0.5 and 1.0 unit/mg of protein.

Protein determination

Protein was determined using a modified Lowry technique as described by Peterson (1977), with bovine serum albumin as a standard. This assay gave protein concentrations for purified HMG-CoA synthase which agreed to within 10% of that determined by amino acid analysis.

Determination of covalently bound [^{14}C]acetyl-enzyme

Covalently bound [^{14}C]acetyl-enzyme was determined as trichloroacetic acid-precipitated protein by the method of Peterson (1977).

Samples (20–50μl) of an incubation of HMG-CoA synthase with [1-^{14}C]acetyl-CoA, containing 5–50μg of protein, were mixed with 1 ml of cold 6% (w/v) trichloroacetic acid. After adding 0.1 ml of 0.15% (w/v) sodium deoxycholate and mixing, the precipitated protein was collected by centrifugation at 10000**g** for 10min. The pellet was washed with 3 × 0.5ml of ice-cold 6% trichloroacetic acid to remove unbound ligand. The protein pellet was then treated as in the Peterson (1977) protein assay and, after reading of the A_{750}, 0.5ml of the protein-containing assay solution was counted for radioactivity.

Radioactivity was measured by a liquid-scintillation method, using 3ml of toluene/Triton X-100 (2:1, v/v), containing 5g of 2,5-diphenyloxazole/litre.

Centrifuge gel-filtration

Protein was separated rapidly from unbound ligand by centrifuge gel-filtration, basically as described by Penefsky (1977). A disposable 1 ml plastic syringe was fitted with a porous polyethylene disc and filled completely with Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden), previously swollen in buffer. Before and after the mixture of protein and ligand (0.1–0.2ml) was applied to the gel surface, the column was centrifuged at 4°C and 100**g** for exactly 2min. The protein was eluted from the column with only minimal contamination by unbound ligands, which were largely retained by the Sephadex.

Determination of CoA

CoA was determined spectrophotometrically by monitoring the conversion of NAD^+ into NADH in the presence of 2-oxoglutarate dehydrogenase complex from *Escherichia coli* (kindly given by Dr. R. N. Perham). The assay contained 50mM-KH_2PO_4/KOH, pH7.5, 1mM-$MgCl_2$, 3mM-NAD^+, 0.2mM-thiamin pyrophosphate, 2mM-2-oxoglutarate and 40μg (0.32 unit) of 2-oxoglutarate dehydrogenase complex in a final volume of 0.7ml, maintained at 30°C. The production of NADH on addition of CoA was measured at 340nm.

Two-dimensional mapping of amino acids

This was performed on cellulose thin-layer plates (Polygram CEL400, 0.1mm layer; Macherey, Nagel and Co.) (10cm × 20cm), as described by Perham (1978). Electrophoresis was performed at pH6.5 in 10% (v/v) pyridine/0.5% (v/v) acetic acid. Mobility on electrophoresis (R_{Asp}) was determined relative to that of aspartic acid.

For the second dimension the dried plate was developed by ascending chromatography at right angles in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.).

Amino acids were detected by spraying with ninhydrin/cadmium stain (Perham, 1978), and radioactive compounds were detected by autoradiography.

Results

Formation of an acetyl-enzyme derivative

Incubation of ox liver HMG-CoA synthase with a 10-fold excess of [1-^{14}C]acetyl-CoA resulted in the rapid formation of covalently bound radioactivity that was stable to trichloroacetic acid precipitation of the protein. This incorporation occurred within 1 min, and when the incubation was performed at 0°C it represented between 0.1 and 0.5 mol of [^{14}C]acetyl groups bound per mol of synthase dimer, depending on the preparation of HMG-CoA synthase. Prolonged incubation at 0°C gave only a slow increase in the amount of [^{14}C]-acetyl groups associated with the protein (Fig. 1*a*). Raising the temperature to 30°C, however, produced a dramatic increase in acetylation within a few minutes (Fig. 1*b*). By performing 10min preincubations at different temperatures between 0 and 30°C, it was found that the fraction of enzyme capable of being rapidly acetylated (i.e. within 1min) increased with temperature up to about 20°C, when it reached a maximum. The maximum stoichiometry of acetylation was found by titrating samples of HMG-CoA synthase with increasing concentrations of [1-^{14}C]acetyl-CoA. Fig. 2 shows results obtained at 30°C by measuring the acid-precipitatable radioactivity, but similar results were obtained when the experiment was performed under non-denaturing conditions by using the equilibrium-binding ultrafiltration method of Paulus (1969).

The stoichiometry at 30°C was found to vary between enzyme preparations (0.6–0.85mol of acetyl

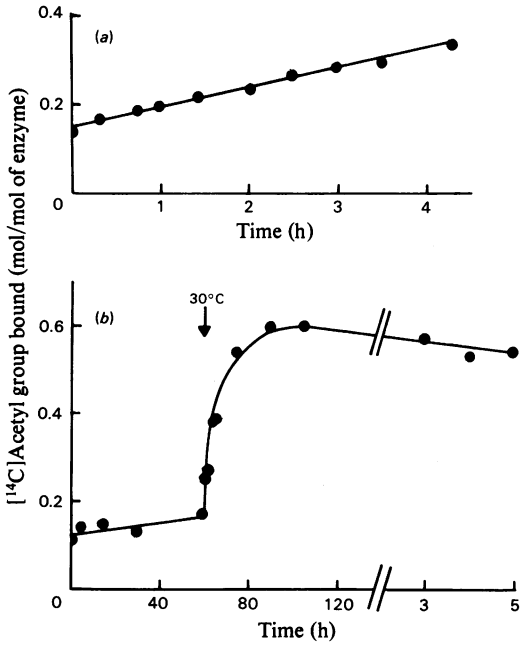

Fig. 1. *Effect of temperature on the formation of acetyl-enzyme*

(*a*) HMG-CoA synthase (0.7mg of protein/ml; 7μM) was equilibrated at 0°C in 0.1M-KH_2PO_4/KOH buffer, pH7. The reaction was started by the addition of [1-^{14}C]acetyl-CoA (5.55Ci/mol) to a concentration of 500μM. Samples (20μl; 140pmol of HMG-CoA synthase protein) were removed at various time intervals and quenched into 1ml of ice-cold 6% (w/v) trichloroacetic acid. The amounts of precipitated protein and protein-bound radioactivity were determined as described in the Experimental section. (*b*) As in (*a*), except that, after 60min of incubation at 0°C, the incubation temperature was rapidly raised to 30°C and the incubation was continued.

group bound/mol of enzyme), but never exceeded 0.85mol of acetyl group bound/mol of synthase dimer. The stoichiometry was not significantly affected by a 100-fold variation in the protein concentration.

To confirm that an acetyl-enzyme derivative of HMG-CoA synthase was being formed, the production of CoA was continuously monitored on mixing substrate quantities of HMG-CoA synthase at various temperatures with a 10-fold excess of acetyl-CoA. These experiments showed a rapid 'burst' of CoA production on mixing, followed by a very much slower steady-state release of CoA. The amount of CoA released in the 'burst' phase from a fixed amount of protein increased with temperature over the same range as was observed by direct acetylation, reaching a similar maximum at 20°C.

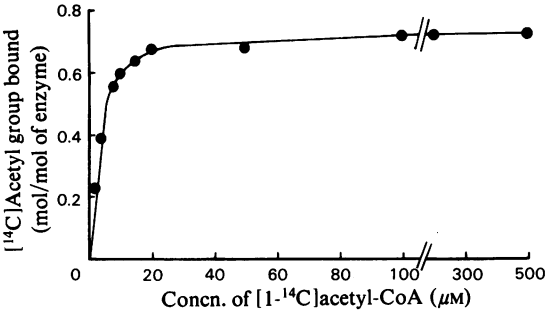

Fig. 2. *Titration of HMG-CoA synthase with [1-^{14}C]-acetyl-CoA at 30°C*

HMG-CoA synthase (0.67mg of protein/ml; 6.7μM) was incubated at 30°C for 1min with various concentrations of [1-^{14}C]acetyl-CoA (4Ci/mol) in a buffer containing 0.1M-KH_2PO_4/KOH, pH7, and 15% (v/v) glycerol. The final incubation volume was 0.05ml and contained 335pmol of enzyme dimer. The reaction was terminated by the addition of 1ml of ice-cold 6% (w/v) trichloroacetic acid. The precipitated protein-bound radioactivity was determined as described in the Experimental section.

Stability of isolated acetyl-enzyme

[^{14}C]Acetyl-HMG-CoA synthase was obtained virtually free from [1-^{14}C]acetyl-CoA that had not reacted by rapid centrifuge gel-filtration of the mixture of HMG-CoA synthase and excess [1-^{14}C]acetyl-CoA. The isolated [^{14}C]acetyl-enzyme showed a slow loss of radioactivity over a period of hours at 0°C and pH7, which appeared to be first-order with a rate constant of about $0.002\,min^{-1}$ ($t_{\frac{1}{2}} = 5.8$h) (Fig. 3*a*). When [^{14}C]acetyl-enzyme was incubated at 30°C and pH7 it was found to be considerably less stable, with a first-order rate constant of $0.1\,min^{-1}$ and a half-life of only 7min (Fig. 3*a*).

Addition of acetoacetyl-CoA (25μM) to isolated [^{14}C]acetyl-enzyme (7.5μM) resulted in 99.9% of the protein-bound radioactivity being lost within 1min. Addition of CoA to isolated [^{14}C]acetyl-enzyme caused very rapid loss of protein-bound radioactivity (Fig. 3*b*), but the extent was dependent on the concentration of CoA (Table 1).

The data for the loss of radioactivity from [^{14}C]-acetyl-enzyme at various concentrations of CoA are consistent with a $K_{eq.} \simeq 10$ for the reaction:

$$E + Ac\text{-}CoA \rightleftharpoons E - Ac + CoA$$

where $$K_{eq.} = \frac{[E\text{-}Ac]\,[CoA]}{[E]\,[Ac\text{-}CoA]}$$

(see Table 1). This was also confirmed by constructing theoretical binding curves for the data in Fig. 2 by using various values of $K_{eq.}$, where a good fit was only obtained if $K_{eq.} > 1$.

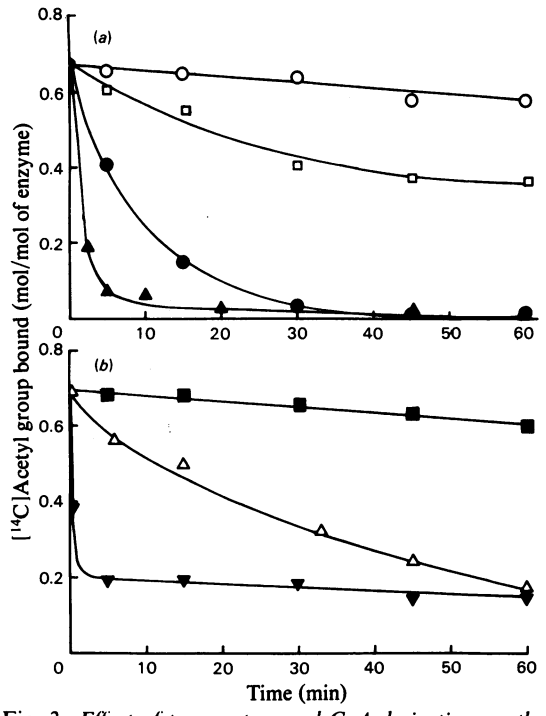

Fig. 3. *Effect of temperature and CoA derivatives on the stability of acetyl-enzyme*

[^{14}C]Acetyl-enzyme was prepared by incubating HMG-CoA synthase (1 mg of protein/ml; 10 μM) with 100 μM-[1-^{14}C]acetyl-CoA (4 Ci/mol) for 2 min at 30°C. Unbound [1-^{14}C]acetyl-CoA was removed by centrifuge gel-filtration in 0.1 M-KH_2PO_4/KOH, pH 7 (see the Experimental section). The resulting [^{14}C]acetyl-enzyme solution was divided up and treated as follows: (*a*) (○) incubated at 0°C; (●) incubated at 30°C; (□) incubated at 0°C plus 100 μM-succinyl-CoA; (▲) incubated at 30°C plus 100 μM-acetyl-CoA; (*b*) (■) incubated at 0°C plus 100 μM-desulpho-CoA; (△) incubated at 0°C plus 100 μM-acetyl-CoA; (▼) incubated at 0°C plus 100 μM-CoA. Samples (25 μl; 250 pmol of HMG-CoA synthase) were removed at various times and quenched into 1 ml of ice-cold 6% (w/v) trichloroacetic acid. The precipitated protein was collected by centrifugation and the amount of protein-bound radioactivity was determined as described in the Experimental section.

The effect of CoA-containing compounds on the stability of acetyl-enzyme is shown in Fig. 3. Contrary to the observation by Middleton & Tubbs (1974), using yeast HMG-CoA synthase, desulpho-CoA did not stimulate hydrolysis of ox liver acetyl-synthase. Acetyl-CoA, and to a lesser extent succinyl-CoA, did stimulate hydrolysis (Fig. 3).

The site of acetylation is a reactive cysteine residue

The [^{14}C]acetyl-HMG-CoA synthase linkage was shown to be acid-stable, since over 90% of the protein-bound acetyl groups were recovered in the pellet when the [^{14}C]acetyl-enzyme, isolated by gel filtration, was precipitated with ice-cold 6% (w/v) trichloroacetic acid.

Treatment of trichloroacetic acid-precipitated [^{14}C]acetyl-enzyme with either 1 M-hydroxylamine, pH 8, or performic acid for 1 h at 20°C resulted in almost complete release of [^{14}C]acetyl groups from the protein. Stability to cold acid and cleavage by hydroxylamine indicate a labile ester (Noda *et al.*, 1953) or amide, but the lability to performic acid strongly suggests a thioester linkage (Lynen, 1967).

The presence of a reactive cysteine residue that is essential to catalytic activity was suggested by the susceptibility of HMG-CoA synthase to inhibition by reagents that react with thiol groups. For example, preincubation of HMG-CoA synthase (8 μM) with methyl methanethiosulphonate (80 μM) caused 80% inhibition of enzyme activity in less than 1 min at 30°C, but in the presence of acetyl-CoA (100 μM) over the same time period, the activity was virtually unaffected.

A titration of HMG-CoA synthase activity with methyl methanethiosulphonate showed a linear correlation between the concentration of inhibitor and the residual enzyme activity (Fig. 4). Extrapolation showed that 1.2 mol of methyl methanethiosulphonate/mol of enzyme dimer was required for complete inhibition of enzyme activity.

Inhibition of HMG-CoA synthase activity was

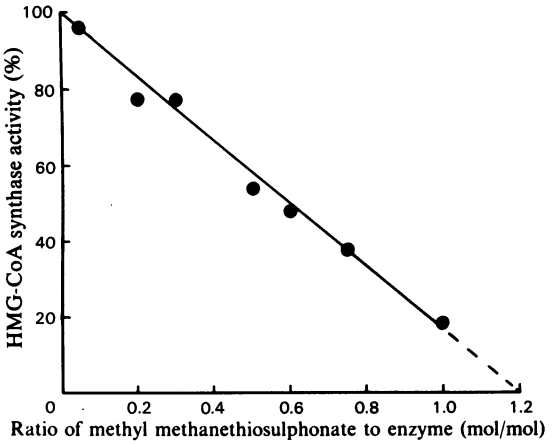

Fig. 4. *Titration of HMG-CoA synthase activity with methyl methanethiosulphonate*

Samples of HMG-CoA synthase (50 μl of 20 μM-enzyme) were incubated at 30°C with different molar ratios of methyl methanethiosulphonate. Samples (10 μl) were taken for assay of HMG-CoA synthase activity after 10 and 20 min to check whether maximum inhibition had been achieved. These assays were generally identical to within 10%. The mean of these two enzyme activities is expressed as a percentage of a control from which methyl methanethiosulphonate was omitted. The enzyme activity of this control remained unchanged over the course of the experiment.

Table 1. *Breakdown of [^{14}C]acetyl-enzyme by CoA*

[^{14}C]Acetyl-enzyme was prepared as described in the legend to Fig. 3 and was mixed with different concentrations of CoA at 0°C. After 10min incubation, the amount of protein-bound radioactivity remaining was determined as described in the Experimental section. The breakdown of [^{14}C]acetyl-enzyme is expressed as a percentage of the original concentration of [^{14}C]acetyl-enzyme. By using the concentrations of [^{14}C]acetyl-enzyme and CoA given, the theoretical percentage breakdown of [^{14}C]acetyl-enzyme was determined for three values of $K_{eq.}$: see the text for definition of $K_{eq.}$.

[CoA] (μM)	Original concn. of [^{14}C]acetyl-enzyme (μM)	Breakdown of [^{14}C]acetyl-enzyme (%)	Predicted breakdown of [^{14}C]acetyl-enzyme (%)		
			$K_{eq.} = 1$	$K_{eq.} = 10$	$K_{eq.} = 100$
35	7.5	40.0	82.4	47.1	19.0
100	3.5	80.3	96.6	78.1	40.8
1300	4.0	95.7	99.7	97.1	80.0

also obtained with bromoacetyl-CoA (Lowe & Tubbs, 1983), *N*-ethylmaleimide and bromopyruvate. When the reaction was performed at 0°C instead of 30°C, however, the inhibition observed with these reagents was biphasic, in that there was an initial rapid loss of activity to about 50% inhibition, followed by a much slower rate of inhibition. This is illustrated for bromoacetyl-CoA in Fig. 5. Many hours were required to achieve complete inhibition at 0°C, whereas by raising the temperature to 30°C the remaining activity was lost within 5min (cf. Fig. 5). This behaviour is similar to that seen for the acetylation of HMG-CoA synthase by [1-^{14}C]acetyl-CoA (Fig. 1), suggesting that the thiol reagents might be reacting at, or near, the site of acetylation. This was confirmed by the experiment described in Fig. 6, in which the extent of inhibition by bromopyruvate was found to be inversely related to the number of acetylation sites remaining.

The nature of the reactive thiol group on HMG-CoA synthase was investigated by using radiolabelled bromoacetyl-CoA as an affinity label for the acetyl-CoA-binding site. This reagent reacted rapidly and stoichiometrically with HMG-CoA synthase to produce a stable alkylated derivative (Lowe & Tubbs, 1983).

Bromo[1-^{14}C]acetyl-CoA-treated HMG-CoA synthase was precipitated with trichloroacetic acid and washed to remove unchanged reagent. It was then hydrolysed with 6M-HCl for 24h at 105°C. Another sample was digested with Pronase (1 part by wt. of Pronase to 10 parts by wt. of protein) for 24h at 37°C. Both of these samples were analysed by electrophoresis at pH6.5, followed by chromatography in the second dimension.

Both acid hydrolysis and Pronase treatment of bromo[1-^{14}C]acetyl-CoA-labelled HMG-CoA synthase produced a single, major, radioactive spot, which had the same mobility on electrophoresis as CM-cysteine ($R_{Asp.} = 0.83$). The position of this spot was exactly superimposable on the position of the hydrolysed radioactive product from the reaction of bromo[1-^{14}C]acetyl-CoA with cysteine (Lowe & Tubbs, 1983) (R_F on chromatography of 0.21), confirming that a cysteine residue had been modified.

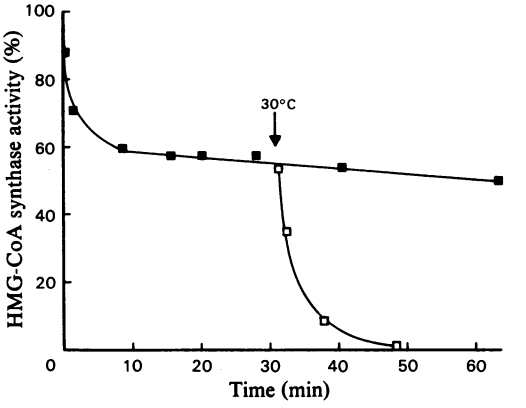

Fig. 5. *Effect of temperature on the inhibition of HMG-CoA synthase activity by bromoacetyl-CoA*

HMG-CoA synthase (8μM) was incubated in 0.1M-KH_2PO_4/KOH, pH7, with 40μM-bromoacetyl-CoA at 0°C (■). At the time indicated (↓), the incubation mixture was divided into two, and half was moved to an incubation temperature of 30°C (□). The remainder was left at 0°C. Samples (20μl) were removed at various times and assayed for HMG-CoA synthase activity. The results are expressed as percentages of a control in which no bromoacetyl-CoA was present.

Digestion of bromo[1-^{14}C]acetyl-CoA-labelled HMG-CoA synthase with trypsin followed by pepsin produced only a single radioactive peptide, as detected by two-dimensional mapping, confirming the specificity of this affinity label for a single site on the enzyme.

Discussion

The observation that an acetyl derivative of ox liver HMG-CoA synthase can be formed is compatible with a reaction pathway involving conden-

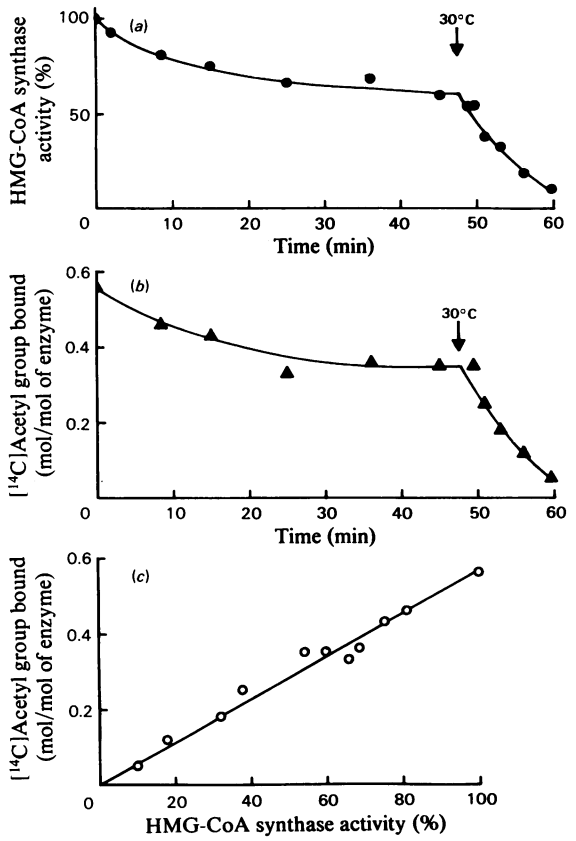

Fig. 6. *Relationship between the extent of inhibition by bromopyruvate and the covalent acetylation of HMG-CoA synthase*

HMG-CoA synthase (0.6mg/ml; 6μM) was incubated with 100μM-bromopyruvate in 0.1M-KH_2PO_4/KOH buffer, pH7, first at 0°C and then, after 47.5min, at 30°C. At intervals throughout the incubation, samples (40μl) were removed and made 1mM in 2-mercaptoethanol by addition of 10μl of 5mM-mercaptoethanol. These samples were left for 5min at 0°C to ensure complete quenching of the bromopyruvate reaction. They were then mixed with 100μM-[1-^{14}C]acetyl-CoA (5.55Ci/mol) and incubated at 30°C for 30min. At the end of this period, 25μl was assayed for HMG-CoA synthase activity. The remaining enzyme was immediately precipitated by the addition of 1ml of ice-cold 6% (w/v) trichloroacetic acid, and was used to determine the amount of [^{14}C]acetyl-enzyme (see the Experimental section). The enzyme activities and the amount of protein-bound [^{14}C]acetyl groups are plotted according to the time of quenching of the bromopyruvate reaction in (*a*) and (*b*), respectively. The correlation between enzyme activity and the incorporation of [^{14}C]acetyl groups is shown in (*c*).

sation of an acetyl-enzyme with acetoacetyl-CoA, as has been previously proposed for the HMG-CoA synthases from yeast and chicken liver (Middleton & Tubbs, 1974; Miziorko & Lane, 1977). The extent of acetylation appears to be highly dependent on temperature, in that rapid acetylation and release of CoA to a maximum value of 0.85mol of acetyl group bound/mol of enzyme dimer was only observed at temperatures greater than 20°C. Below this temperature the extent of acetylation was lowered (cf. Fig. 1). This effect of temperature is similar to that reported for chicken liver HMG-CoA synthase (Miziorko *et al.*, 1976), although it was not observed with yeast HMG-CoA synthase (Middleton & Tubbs, 1974). It can be interpreted in terms of a temperature-dependent conformational change from an inactive to an 'active' form which can be acetylated by acetyl-CoA. This change is rapid and does not involve subunit dissociation, since the enzyme has the relative molecular mass of the dimer at both 4°C and 20°C (Lowe & Tubbs, 1985).

The maximum extent of acetylation at 30°C approaches, but does not exceed, 1.0mol of acetyl group bound/mol of enzyme dimer. Since the enzyme is a dimer of apparently identical subunits (Lowe & Tubbs, 1985), it might be expected that there would be one acetyl-group-binding site per monomer and, hence, two per dimer. Trivial reasons for the observed stoichiometry being lower than the actual number of acetylation sites, such as the presence of inactive protein or inaccuracies in measuring the protein concentration, could account for the small differences in the stoichiometry measured for different preparations of HMG-CoA synthase. They are unlikely, however, to explain why a stoichiometry of anything approaching 2 acetyl groups bound per enzyme dimer was never seen, either by trichloroacetic acid precipitation of the covalent acetyl-enzyme derivative or under the non-denaturing conditions used to measure the 'burst' release of CoA and the direct equilibrium binding of [^{14}C]acetyl-CoA.

The maximum stoichiometries of acetylation for yeast and chicken liver HMG-CoA synthases have been reported as 0.5 and 1.0mol of acetyl group bound/mol of enzyme, respectively (Middleton & Tubbs, 1974; Miziorko *et al.*, 1976). The subunit composition of yeast synthase is not yet established, but the avian synthase is also dimeric (Reed *et al.*, 1975; Clinkenbeard *et al.*, 1975). Thus, for all three HMG-CoA synthases, the amount of acetylation observed was not more than 0.5mol of acetyl group bound per mol of polypeptide chain. Hence a form of 'half-of-the-sites' reactivity may be inherent in the mechanism of HMG-CoA synthases.

Stoichiometries close to 1mol/mol of enzyme dimer were also observed when the enzyme activity was titrated with thiol-specific reagents, such as methyl methanethiosulphonate (Fig. 4) or the active-site-directed irreversible inhibitor bromo[1-^{14}C]acetyl-CoA (Lowe & Tubbs, 1983). Acetyl-

CoA protects against inactivation by both of these reagents, although this protection is not complete, presumably owing to turnover of the acetyl-enzyme (cf. Fig. 3*a*). The extent of inactivation also shows the same dependence on temperature (Figs. 5 and 6) as was observed for the extent of acetylation (Fig. 1). Such observations confirm that these reagents are reacting specifically at a single site on the enzyme dimer, which is probably the site of acetylation. As with acetylation (see above), the temperature-dependence of the extent of inactivation suggests the presence at 0°C of two forms of the enzyme, only one of which is susceptible to inhibition by thiol-specific reagents. The division between the rapid and slow phases of inactivation of HMG-CoA synthase (Figs. 5 and 6) probably represents the position of a conformational equilibrium, which shifts towards the form of the enzyme that can react with thiol-specific reagents as the temperature is raised to 30°C.

Isolated acetyl-enzyme is relatively stable at 0°C ($t_{\frac{1}{2}} = 5.8$ h), although considerably less so at 30°C ($t_{\frac{1}{2}} = 7$ min) (Fig. 3*a*). This suggests that the same conformational change that affects the enzyme's ability to be acetylated and to perform the overall enzyme reaction also affects its ability to act as a slow acetyl-CoA hydrolase. This hydrolase activity is thought to mimic the hydrolysis of the HMG-CoA-enzyme intermediate in the normal enzyme reaction (Middleton & Tubbs, 1974; Miziorko *et al.*, 1975). The hydrolysis of acetyl-enzyme is stimulated by the presence of acetyl-CoA and to a lesser extent by succinyl-CoA (Fig. 3), but not by desulpho-CoA, the last observation being in contrast with that with yeast HMG-CoA synthase (Middleton & Tubbs, 1974). Acetyl-CoA would not be expected to react chemically with the acetyl-enzyme, and therefore must be acting catalytically. This would indicate that a complex with acetyl-CoA bound to acetyl-enzyme might exist, having 2 mol of acetyl group bound per mol of enzyme. This was not, in fact, observed in equilibrium binding experiments, and acetyl-CoA gives only poor substrate inhibition (Lowe & Tubbs, 1985), suggesting that this second molecule of acetyl-CoA might bind only very weakly. Succinyl-CoA is known to act as an analogue of acetyl-CoA (D. M. Lowe & P. K. Tubbs, unpublished work), and is, thus probably mimicking the effect of acetyl-CoA.

The finding that $K_{eq.} \simeq 10$ for the acetylation of ox liver HMG-CoA synthase by acetyl-CoA contrasts with the value of $K_{eq.} \simeq 1$, determined for yeast and chicken liver synthases (Middleton & Tubbs, 1974; Miziorko *et al.*, 1975). Thus, although for yeast and chicken liver HMG-CoA synthases the acetylation reaction is readily reversible, for ox liver synthase the equilibrium favours the formation of acetyl-enzyme.

The work presented here on the specific labelling of a cysteine residue by the substrate analogue bromo[1-^{14}C]acetyl-CoA (Lowe & Tubbs, 1983), the chemical structure of the acetyl-enzyme and inhibitor studies strongly suggest that the site of acetylation of ox liver HMG-CoA synthase is a cysteine thiol group. Work on the chicken liver HMG-CoA synthase has also identified a cysteine residue as the site of acetylation (Miziorko *et al.*, 1975), and chemical evidence for yeast HMG-CoA synthase strongly supports the presence of a thioester linkage in the acetyl-enzyme (Middleton & Tubbs, 1974). It should be of interest to use bromo-[1-^{14}C]acetyl-CoA to label peptides containing this cysteine residue so that the region around the site of acetylation from these different sources can be compared.

This work was supported by a grant and studentship (D. M. L.) from the Medical Research Council, London.

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