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The Molecular Weight and Thiol Residues of Acetyl-Coenzyme A Synthetase from Ox Heart Mitochondria

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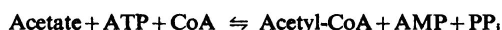
1. A constant molecular weight of 57000 was obtained by gel filtration of highly purified acetyl-CoA synthetase over a 1000-fold range of enzyme concentrations. The amino acid analysis is reported. 2. With native enzyme at 20°C the relatively rapid reaction of four thiol residues with *p*-hydroxymercuribenzoate caused an immediate inhibition reversible by either CoA or mercaptoethanol. Other substrates did not protect against this rapid inhibition. 3. The much slower reaction of the remaining four thiol residues was independent of the concentration of the mercurial, first-order with respect to enzyme, and had a large energy of activation (+136kJ/mol), suggesting that a conformation change in the protein was rate-limiting. This slow phase of the reaction was accompanied by an irreversible inactivation of the enzyme. 4. The effects of substrates on this irreversible inactivation at pH7.0 in 5mM-$MgCl_2$ indicated strong binding of ATP and pyrophosphate by the enzyme (concentrations for half-maximal effects, $K_{\frac{1}{2}}$, were <30μM and <10μM respectively) and weaker binding of acetyl-CoA ($K_{\frac{1}{2}}$ about 1mM), AMP ($K_{\frac{1}{2}}$ about 2mM) and acetate. In the presence of acetate, $MgCl_2$ and *p*-hydroxymercuribenzoate, titration of the enzyme with ATP revealed at least two ATP binding sites/mol. 5. The experiments suggest that reaction of the thiol residues with mercurial causes loss of enzymic activity by altering the structure of the enzyme, rather than that the thiol residues play a direct role in the catalysis.

Acetyl-CoA synthetase (EC 6.2.1.1) catalyses the reaction:



Acetate + ATP + CoA $\rightleftharpoons$ Acetyl-CoA + AMP + PP_i

Although much work has been done on the mechanism of the enzymic catalysis [for reviews see Jencks (1962) and Loftfield (1971)], the instability of the enzyme has hindered its purification and the investigation of its physical properties and amino acid composition. The crystalline enzyme obtained from heart mitochondria (Webster, 1965) was unstable and the behaviour of fresh preparations changed over a period of a few days, the enzyme showing an increasing tendency to aggregate. To our knowledge, no work has yet been done on the equilibrium binding of substrates to acetyl-CoA synthetase. It has been suggested that thiol groups are directly involved in the catalytic activity of other AMP-forming ligases (Lee & McElroy, 1969; Loftfield & Eigner, 1969), but Sharkova (1968) found no effect of *p*-hydroxymercuribenzoate on the enzyme from rabbit heart.

In the present paper a new purification procedure is described which gives 70% greater specific activity than the best previously reported (Webster, 1965). The activity of the new preparation is stable for days at 4°C and for months at −70°C. The protein shows little tendency to aggregate. The molecular weight and amino acid analyses are reported. The purity and stability of the enzyme seemed adequate for investigating both the binding of substrates and the effect on enzymic activity of modifying the cysteine residues with *p*-hydroxymercuribenzoate.

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Experimental Procedures

Materials

CoA (96% pure) and acetyl-CoA were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Malate dehydrogenase and citrate synthase were from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. Other coenzymes and proteins, *p*-hydroxymercuribenzoate and 5,5′-dithiobis-(2-nitrobenzoic acid) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Potassium pyrophosphate was from K & K Laboratories, Plainview, N.Y., U.S.A., TEAE-cellulose was from the Brown Co., Berlin, N.H., U.S.A., and Sephadex gels and Blue Dextran 2000 were from Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A. Tris base and ammonium sulphate (special enzyme grade) were from Mann

Research Laboratories Inc., New York, N.Y., U.S.A. Other salts and buffers were reagent-grade chemicals.

Methods

Protein concentrations. These were determined from their E_{280} (in Tris–HCl buffer, pH 8.3) or by the biuret reaction (Gornall *et al.*, 1949). An increase in E_{540}^{1cm} of 0.1/mg of protein in the 3 ml biuret assay mixture was assumed. Dry weights were determined on three independent preparations of enzyme, which were purified through the second gel-filtration step and dialysed exhaustively against 5 mM-$KHCO_3$. Samples, containing 2–8 mg of protein, were dried in air at 100°C together with dialysis-fluid blanks. When weighed immediately after removal from the oven, the dry mass was 94.5±1% of the protein content determined by biuret assay. On highly purified material, the ϵ_{280} was 1.41 ml·mg^{-1}·cm^{-1}.

Enzyme assays. As a routine acetyl-CoA synthetase activity was measured in the forward direction by observing NADH production spectrophotometrically in the standard coupled assay with malate dehydrogenase and citrate synthase (Hele, 1954; Pearson, 1965). Reaction mixtures of 1 ml contained: 100 mM-Tris–HCl buffer, pH 8.3, 3.6 mM-$MgCl_2$, 6 mM-potassium acetate, 3 mM-ATP (potassium disodium salt), 2 mM-CoA, 8 mM-L-malate (potassium salt), 1 mM-NAD^+, 0.1 mM-NADH, 10 μg of malate dehydrogenase and 20 μg of citrate synthase. The unit of activity is defined as the amount of enzyme required to produce 1 μmol of NADH/min at 30°C.

The reverse reaction was measured directly by monitoring cleavage of the thioester bond of acetyl-CoA; a value of 4.3 cm^{-1} was used for the millimolar extinction coefficient of the decrease in absorption at 232 nm. Reaction mixtures (1 ml) contained 100 mM-Tris–HCl buffer, pH 8.0, 100 mM-KCl, 2 mM-$MgCl_2$, 2 mM-potassium pyrophosphate, 0.1 mM-acetyl-CoA and 0.1 mM-5′-AMP. With about 2 μg of acetyl-CoA synthetase, the decrease in extinction was linear with time for several minutes, and the rate was about 2.5% of the rate of the forward reaction in the standard coupled assay at the same temperature.

Enzyme purification. Steps 1–3. Acetyl-CoA synthetase was isolated from ox heart mitochondria and purified through the first ammonium sulphate fractionation as previously described (steps 1–3 in Webster, 1969). The precipitate so obtained was dissolved in 10 mM-$KHCO_3$–3 mM-mercaptoethanol to about 65 mg of protein/ml, thoroughly dialysed against the same buffer, and stored at −70°C. All subsequent operations were performed at 0–4°C, and 3 mM-mercaptoethanol was included in all buffers. In ammonium sulphate fractionations the pH was kept near 8.0 by additions of 1 M-NH_3.

Step 4. Material from 10 kg of heart muscle was thawed and diluted with water to 10 mg of protein/ml. Potassium pyrophosphate and $MgCl_2$ were added to give final concentrations of 0.5 mM, and the pH was adjusted to 5.0 by slow addition of 1 M-formic acid. The mixture was promptly centrifuged at 20000 *g* for 5 min, and the precipitate suspended in about 20 ml of water and adjusted to pH 8 with aq. NH_3. Insoluble material was removed by centrifugation, and the supernatant solution diluted with water to 25 mg of protein/ml. Some material absorbing at 260 nm was removed by the slow addition of an aq. 3% (w/v) solution of neomycin sulphate (not more than 50 μg/mg of protein) until little further precipitate formed, while the pH was kept at 7.8 by addition of aq. NH_3. The precipitate was removed by centrifugation at 27000 *g* for 15 min, and solid KCl and $KHCO_3$ were added to the supernatant solution to concentrations of 100 mM and 20 mM respectively.

Step 5. To each 1 ml of supernatant solution from Step 4, 0.43 g of $(NH_4)_2SO_4$ was added, then the precipitate was collected by centrifugation at 27000 *g* for 20 min. This precipitate was extracted successively with 20 mM-$KHCO_3$ containing 2.15 M-, 1.55 M- and 1.35 M-$(NH_4)_2SO_4$ in turn. For each extraction the precipitate was suspended in 40 ml of the appropriate buffer, stirred for 10 min, and then centrifuged at 27000 *g* for 15 min. The supernatant solutions obtained at 1.55 M- and 1.35 M-$(NH_4)_2SO_4$ were combined. The protein was precipitated from them by the addition of 0.24 g of $(NH_4)_2SO_4$/ml, collected by centrifugation, and dissolved in a small volume of 20 mM-$KHCO_3$ and stored at −70°C.

Step 6. Thawed material from Step 5, derived from 20 kg of minced heart, was dialysed for 2 h against 10 mM-$KHCO_3$ and diluted with the same buffer to 40 mg of protein/ml. The solution (<7 ml) was applied to a column (32 cm × 2.5 cm) of Sephadex G-100 equilibrated with 20 mM-$KHCO_3$. The column was eluted with the same buffer at a flow rate of 20 ml/h. Fractions, from the second protein peak, with a specific activity above 9 units/mg, were combined.

Step 7. Material from Step 6 was run at 15 ml/h into a column (19 cm × 0.9 cm) of TEAE-cellulose equilibrated with 10 mM-$KHCO_3$. The column was developed with a linear gradient by running 160 ml of 10 mM-$KHCO_3$, containing 0.3 M-KCl, into 160 ml of 10 mM-$KHCO_3$. The maximum specific activity occurred immediately after the main protein peak, and thereafter the specific activity and E_{280}/E_{260} ratio gradually decreased with the protein concentration. Active fractions of about 2 ml each were stored separately at −70°C.

Purification results are shown in Table 1. In most experiments, TEAE-cellulose eluates of specific activity greater than 36 units/mg were used. Occasionally these were further purified to remove a small

Table 1. *Purification of acetyl-CoA synthetase from ox heart mitochondria*

Mean results from a large number of preparations are normalized for an initial 20kg of minced muscle. Protein concentrations were determined by the biuret reaction in steps 1 to 4 inclusive, and by the E_{280} in subsequent steps.

Step	Volume (ml)	E_{280}/E_{260}	Protein (mg/ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Mitochondrial extract	8000	—	2.5–4.0	3400	0.17	Variable
After first $(NH_4)_2SO_4$	120	—	60	3000	0.42	(100)
After pH and neomycin	67	—	18	2400	2.0	80
After second $(NH_4)_2SO_4$	4.0	1.5	57	1440	6.3	48
After Sephadex G-100	39	1.6	1.7	905	13.5	30
Consecutive fractions from	2.6	1.91	0.87	90	41	3.0
TEAE-cellulose	7.2	1.75	0.6–0.8	210	37–41	7.0
chromatography	13.0	1.70	0.4–0.6	180	24–36	6.0

amount of yellow colour and a high-molecular-weight impurity. About 20mg of enzyme was then concentrated by ammonium sulphate precipitation, dissolved in a small volume of 10mM-$KHCO_3$, applied to a column (15cm×0.9cm) of Sephadex G-100, and eluted with 20mM-$KHCO_3$. A small rise in specific activity resulted, but the enzyme was now very unstable: 40% of the catalytic activity was lost during the next 24h at 4°C, and changes in the structure of the protein occurred (see the Results section).

Molecular-weight determinations by gel filtration. These were done by the method of Andrews (1965) at 4°C in 20mM-Tris–HCl, pH8.0, containing 130mM-KCl, -NaCl or -tetramethylammonium chloride. Proteins and Dextran Blue in 1ml of the appropriate buffer were individually applied to a column of gel (50cm×2.3cm). Fractions of equal volume (approx. 3ml) were collected at a flow rate of 20ml/h. Protein in the effluent was estimated by E_{230} measurements. Elution volumes (V_e) are expressed as ratios to the Dextran Blue exclusion volume (V_0).

Ultracentrifugal analysis. Enzyme solutions were dialysed against three changes of 20mM-Tris–HCl buffer, pH8.0, containing 130mM-KCl for 24h, and then centrifuged at 20000g for 15min before analysis in the Spinco model E analytical ultracentrifuge. Sedimentation-equilibrium experiments were performed by the short-column method of Van Holde & Baldwin (1958). A value of 0.734 for the partial specific volume was calculated from the amino acid analysis by the method of Cohn & Edsall (1943).

Analytical disc-gel electrophoresis. This was performed by the method of Davis (1964) under conditions (pH8.8, β-alanine buffer) described by Killenberg *et al.* (1971).

Amino acid analysis. Tryptophan was measured by the acid–ninhydrin procedure of Gaitonde & Dovey (1970), and by the method of Beaven & Holiday (1952). Cysteine was determined on enzyme freed of mercaptoethanol (see below), by the procedure of Ellman (1959) and by titration with *p*-hydroxymercuribenzoate by the method of Boyer (1954). Stock solutions of about 2mM-*p*-hydroxymercuribenzoate were prepared every few days in 5mM-Tris–sulphate buffer, pH8.0, centrifuged, and standardized by E_{232} measurements in phosphate buffer, pH7.0 ($\epsilon = 16.7$ litre·mmol^{-1}·cm^{-1}).

The remaining amino acids were determined by conventional amino acid analysis by Analytical Bio Chemistry Laboratories Inc., Columbia, Mo., U.S.A., who also determined the tryptophan content after organic acid hydrolysis (Liu & Chang, 1971). Material purified through the second gel-filtration step was adjusted to a concentration of 6M-HCl and hydrolysed *in vacuo* at 110°C for 10, 72 and 90h, or at 145°C for 4h. Samples were then dried, dissolved in the appropriate buffer and applied to the analyser column. Results obtained at 110°C and 145°C agreed within 4% except for methionine, histidine and arginine. The lower content of methionine found after hydrolysis at 145°C was ignored because it presumably indicated destruction at the higher temperature. However, the significantly higher results obtained at 145°C for histidine and arginine are reported.

Inactivation of the enzyme by p-hydroxymercuribenzoate. This was studied by incubating the enzyme with the inhibitor and testing 10 or 20μl portions of the incubation mixture at suitable time-intervals in either the standard coupled assay or the reverse reaction assay. Incubation mixtures, containing buffer and any additives in 0.8ml, were brought to the desired temperature before addition of enzyme. The inactivation was started by addition of inhibitor 1min after the enzyme.

Rate of mercaptide formation between p-hydroxymercuribenzoate and enzyme thiol residues. This was followed by continuous recording of the change in E_{255} with a Gilford model 2400 spectrophotometer. Reaction mixtures (1ml) without inhibitor were

made up in silica cuvettes (1 cm path length) and adjusted to the desired temperature in the thermostatically controlled cell compartment of the spectrophotometer. Reactions were then started by the rapid manual addition of 10 or 20 μl of inhibitor to the cuvette and mixing by inversion; the time-interval between inhibitor addition and the first reliable measurement was about 0.2 min. Before use, the enzyme for these experiments (TEAE-cellulose eluates of specific activity 41 units/mg) was dialysed against 0.1 M-Tris–HCl buffer, pH 8.3, containing 0.2 M-KCl, until no mercaptoethanol could be detected in the enzyme samples by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) in 0.1 M-potassium phosphate buffer, pH 7, at 20°C (under these conditions, the thiol residues of the enzyme react much more slowly than does the mercaptoethanol). During the dialyses, however, 10–20% of the enzymic activity was lost (see the Discussion section).

Results

Purity

Protein from TEAE-cellulose eluates approached homogeneity as judged by its behaviour on gel filtration and in the ultracentrifuge (see below); however, a faint yellow colour and a high-molecular-weight component were still present. One sharp leading and several blurred trailing bands were observed on analytical disc-gel electrophoresis of either the protein from TEAE-cellulose or material further purified by gel filtration. However, if an excess of acetyl adenylate was added to either preparation immediately before electrophoresis, a single discrete protein band resulted.

Molecular weight by gel filtration

In Fig. 1 are shown elution profiles for acetyl-CoA synthetase from Sephadex G-100 for samples at initial protein concentrations between 8.6 mg/ml and 7.6 μg/ml. Recovery of activity was 64% at the lowest concentration and up to 90% at higher concentrations. The exclusion volume did not significantly change with the protein concentration. Protein and activity curves were essentially coincident, although a distinct shoulder in the activity curve, corresponding to a molecular weight of 100000, was frequently observed. No significant amount of protein or activity was eluted after ovalbumin (45000 molecular weight).

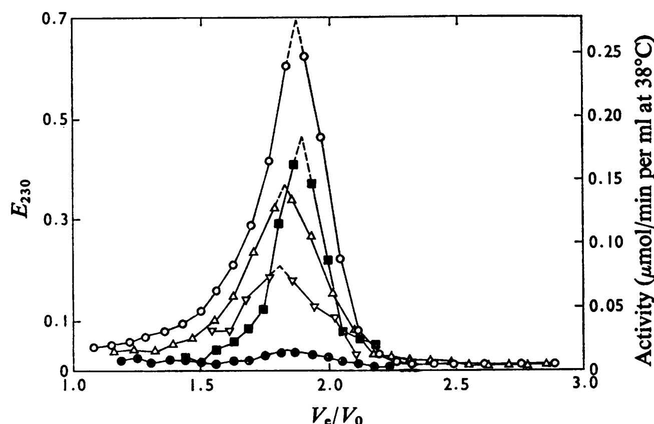

Fig. 1. *Elution profiles from Sephadex G-100 for acetyl-CoA synthetase over a 1000-fold range of initial protein concentrations*

Samples of 1 ml were applied to the column and eluted with 20 mM-Tris–HCl buffer, pH 8.0, containing 130 mM-KCl. At an initial protein concentration of 8.6 mg/ml, the E_{230} divided by 3 of the eluate fractions is shown (○), and at 920 μg/ml, the E_{230} (△); at 100 μg/ml, the E_{230} (●) and enzymic activity (■); and at 7.6 μg/ml, the activity multiplied by 10 (▽). Molecular weights and relative elution volumes (V_e/V_0) for marker proteins were: yeast alcohol dehydrogenase, 149000 and 1.25 (molecular weight from Dickinson, 1970); bovine serum albumin dimer, 135000 and 1.24; bovine serum albumin monomer, 67500 and 1.69; ovalbumin, 45000 and 2.09; chymotrypsin, 24500 and 2.51; horse heart cytochrome *c*, 12400 and 2.91 (molecular weights compiled by Andrews, 1965).

Substitution of NaCl or tetramethylammonium chloride for KCl in the buffer caused no change in the relative exclusion volume, although the activities of acetyl-CoA synthetase in 20mM-Tris–HCl, pH8.0, containing 130mM of these chlorides were in the proportions K^+:Na^+:Me_4N^+ 100:1:20 (assayed in the presence of 2mM-CoA, 3.6mM-$MgCl_2$, 3mM-ATP and 6mM-acetate).

Some experiments were also conducted with Sephadex G-150 and with an aged sample of Sephadex G-100, which showed a much more sigmoid calibration curve than the fresher gel, but no significant difference in the apparent molecular weight was observed. The mean value for the molecular weight from a total of 15 experiments, at a variety of protein concentrations on Sephadex G-100 and G-150, was 57000, with a range of ±3500.

Ultracentrifugation

Sedimentation-velocity experiments at 20°C with acetyl-CoA synthetase at 4.1 mg/ml, 2.5 mg/ml and 0.84 mg/ml showed a major component with $s_{20,w}$ of 4.84S, 4.83S and 4.78S respectively, and a small amount (about 4%) of heavier material with $s_{20,w}$ of 9.2S. A high value for $\bar{M}_z$ of 83000 was obtained with this material by sedimentation equilibrium, presumably because of the heavy component. After further purification of the enzyme by a second gel-filtration step, as described in the Experimental Procedures section, the 9.2S component could not be seen in sedimentation-velocity experiments. However, the major peak was now skewed to the heavy side, and had a sedimentation coefficient of 5.7S at 20°C. In a sedimentation-equilibrium experiment with this material (1 mg/ml), the plot of $(1/r)(dn_c/dr)$ versus n_c was convex to the n_c axis. A value for $\bar{M}_z$ of 72000 was obtained from the limiting slope near the meniscus.

Amino acid analysis

With 'reagent b' of Gaitonde & Dovey (1970) the colour production with acetyl-CoA synthetase corresponded to 8.9±0.7 mol of internal and *C*-terminal tryptophan/57000*g* of protein. The small amount of colour produced with 'reagent a', which is preferential for *N*-terminal tryptophan, could all be accounted for by this amount of non-*N*-terminal tryptophan. After correction for light-scattering by extrapolation to shorter wavelengths of the small extinctions between 370nm and 320nm, the extinction of 0.193mg of acetyl-CoA synthetase/ml in 0.1M-KOH was 0.219 at 294.4nm and 0.250 at 280nm. From these values 8.5 mol of tryptophan and 18.7 mol of tyrosine were calculated/57000g of protein. The former result agrees well with the value of 8.3 mol of tryptophan obtained by organic acid hydrolysis.

The total amino acid analysis is shown in Table 2. The sum of the mass of anhydro-amino acid residues found accounted for 93.5% of the dry weight. No amino sugars were detected on the chromatograms of material hydrolysed for 10h at 110°C (<0.3mol/57000g of protein). The possible presence of neutral sugars and sialic acids was examined by applying the anthrone and resorcinol tests to the unhydrolysed protein as described by Spiro (1966), but neither was detected (<1 mol/57000g of protein).

Table 2. *Amino acid analysis of acetyl-CoA synthetase*

Unless otherwise indicated, the values (mol/57000g dry weight of protein) are means of chromatographic determinations after hydrolyses for 72 and 90h at 110°C. The results for serine and threonine are for hydrolysis at 110°C extrapolated to zero time of hydrolysis. The result for tryptophan is the mean of u.v., acid–ninhydrin and organic-hydrolysis analyses. The results for histidine and arginine are the mean ± range of results at 110°C and 145°C. Half-cystine was determined by Ellman's (1959) reaction and titration with *p*-hydroxymercuribenzoate.

Aspartic acid + Asparagine	42	Methionine	9.3
Threonine	33	Isoleucine	22
Serine	30	Leucine	44
Glutamic acid + Glutamine	44	Tyrosine	20
Proline	26	Phenylalanine	16
Glycine	46	Tryptophan	8.5
Alanine	42	Lysine	24
Valine	40	Histidine	11.5±1.5
		Arginine	24 ±3
		Half-cystine	8

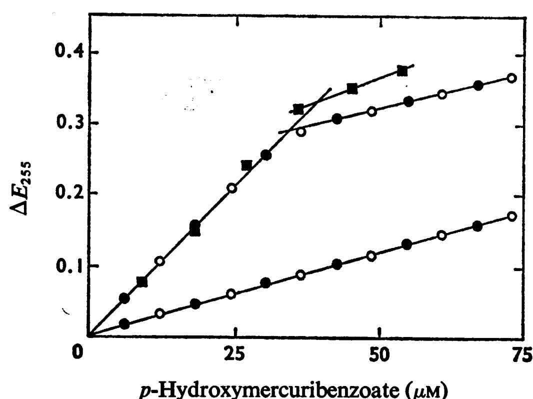

Fig. 2. *Titration of acetyl-CoA synthetase with p-hydroxymercuribenzoate*

Duplicate titrations (○ and ●) in 80mM-Tris–HCl buffer, pH7.7, containing 50mg of sodium dodecyl sulphate/100ml, are shown in the presence and absence of 0.25mg of enzyme/ml. The titration (■) of 0.30mg of enzyme/ml in 0.1M-potassium phosphate buffer, pH8.0, is also shown. The extinction is not corrected for the small dilution occurring at each titration step.

Titration of enzyme with p-hydroxymercuribenzoate

In Fig. 2 are shown duplicate titrations of acetyl-CoA synthetase with *p*-hydroxymercuribenzoate in Tris–HCl buffer, pH7.7, containing sodium dodecyl sulphate at room temperature (about 25°C). Under these conditions the E_{255} became constant in a few minutes at each step of the titration. From the break in the titration plot, this sample of enzyme was found to contain 7.90mol of thiol/mol of protein (57000g). Also shown is the titration of enzyme in 0.1M-potassium phosphate, pH8.0, in which the enzyme was quite stable. Up to 2h was required for the extinction readings to become constant, resulting in less precision than in the previous experiment, but essentially the same value (7.56mol) was obtained. From the values in Fig. 2 and other similar results, ϵ_{255} for the formation of enzyme–mercaptide was calculated to be 6.38 litre·mmol of thiol^{-1}·cm^{-1}. It did not change between pH7.0 and pH8.0, and was not altered by the presence of sodium dodecyl sulphate.

Reaction of enzyme with 5,5′-dithiobis-(2-nitrobenzoate)

In 0.1M-potassium carbonate, pH9.0, in which the enzyme rapidly lost catalytic activity, 7.7 thiol residues/mol of protein were found by reaction with a large excess of the reagent. The process was apparently first-order over at least 90% of the reaction (Fig. 3). In 0.2M-potassium phosphate, pH8.0, containing 4.6M-guanidine hydrochloride, the reaction was complete within 10min. The thiol content decreased when the enzyme was kept in guanidine before addition of dithiobis(nitrobenzoate). This disappearance was second-order with respect to thiol over 70% of the reaction ($k = 328\text{M}^{-1}\cdot\text{min}^{-1}$ at 25°C), and was greatly diminished by 5mM-EDTA. In the presence of EDTA, 7.04mol of thiol/mol of protein was found, compared with 7.75 by extrapolation to zero time of the results obtained in the absence of EDTA.

Kinetics of the reaction with p-hydroxymercuribenzoate

In Fig. 4 are presented the results of experiments at pH8.6 and 20.5°C in which the rate of reaction between enzyme thiol groups and several concentrations of mercurial was measured. An ϵ_{255} of 6.38 litre·mmol^{-1}·cm^{-1} was used for mercaptide formation (see above). The overall reaction was at least biphasic. Approximately 4 thiol groups reacted relatively rapidly at a rate which increased with the concentration of mercurial. The much slower reaction of the remaining residues was independent of the mercurial concentration, provided the latter was in excess. This slow reaction was approximately first-order with a rate constant of about $5.0\times10^{-3}\text{min}^{-1}$.

The effect of temperature and pH on both phases of the reaction of enzyme with *p*-hydroxymercuribenzoate are shown in Fig. 5. At 30.5°C, lowering the pH from 8.4 to 7.1 significantly slowed the rapid phase of the reaction, but after 10min and reaction of 4–5 thiol groups, the process became approximately first-order and proceeded at a rate very close to that at pH8.4. At pH8.4, increasing the temperature from 20.5° to 39.0°C caused a small increase in the rate of the fast phase of the reaction, but dramatically increased the rate of reaction of the last 4 thiol groups. The first-order rate constant for the latter phase of the process increased from $5\times10^{-3}\text{min}^{-1}$ at 20.5°C to $250\times10^{-3}\text{min}^{-1}$ at 39.0°C.

Inhibition of acetyl-CoA synthetase activity by p-hydroxymercuribenzoate

Treatment of acetyl-CoA synthetase with the mercurial caused two types of inhibition, which appeared to be associated respectively with the fast and slow phases of the reaction of the protein thiol groups with the inhibitor. An inhibition reversible by millimolar concentrations of thiols, such as CoA or mercaptoethanol, was examined by measuring the activity of the enzyme in the reverse reaction (acetyl-CoA disappearance). In Fig. 6 are shown the results of experiments in which enzyme was incubated with the mercurial and then tested by this assay. Large

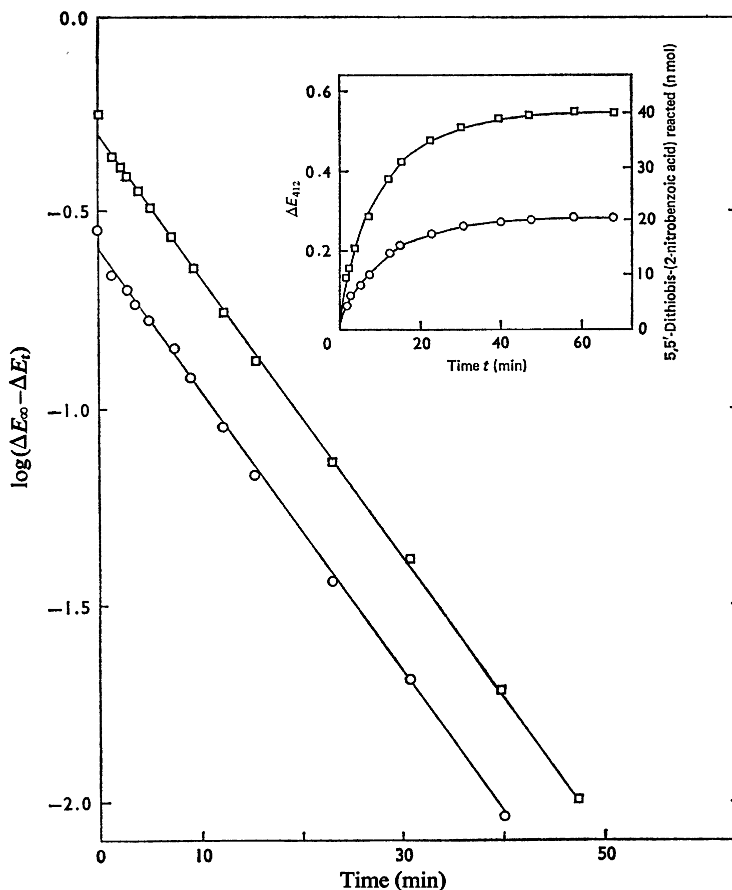

Fig. 3. *Reaction of acetyl-CoA synthetase with* 0.2*m*M-5,5′-*dithiobis*-(2-*nitrobenzoic acid*) *in* 0.1M-K_2CO_3, *pH*9.0, *at* 25°*C*

The inset shows the increase in E_{412} with 0.30mg (□) and 0.15mg (○) of enzyme/ml, corrected by subtraction of the small but continuous increase occurring in the absence of protein. From the final changes (ΔE_{∞}) a thiol content of 7.7mol/57000g of enzyme was calculated. In the main diagram the same results are presented as a first-order reaction plot.

inhibitions were caused by the reaction of less than 4 and less than 2 enzyme thiol groups (curves *b* and *c* respectively). The rapid and complete inhibition observed when enzyme was incubated with an excess of the mercurial (curve *a*) still occurred within 0.7min when the incubation was done at 0°C, and could not be overcome by including in the incubation mixtures 5mM-$MgCl_2$ together with AMP or ATP in the presence or absence of 10mM-acetate. Further, addition of 90μM-*p*-hydroxymercuribenzoate to reverse reaction assay mixtures 3min after enzyme addition caused the reaction to stop immediately (i.e. within 0.4min). None of these inhibitions could be explained by mercaptide formation between the mercurial and CoA produced in the reverse reaction, although such mercaptide formation would decrease the apparent ϵ_{232} for thioester cleavage.

Irreversible inactivation by p-hydroxymercuribenzoate

If the enzymic activity after treatment with *p*-hydroxymercuribenzoate was measured in the standard coupled assay, which contained 2mM-CoA, or in the reverse assay with the additional presence

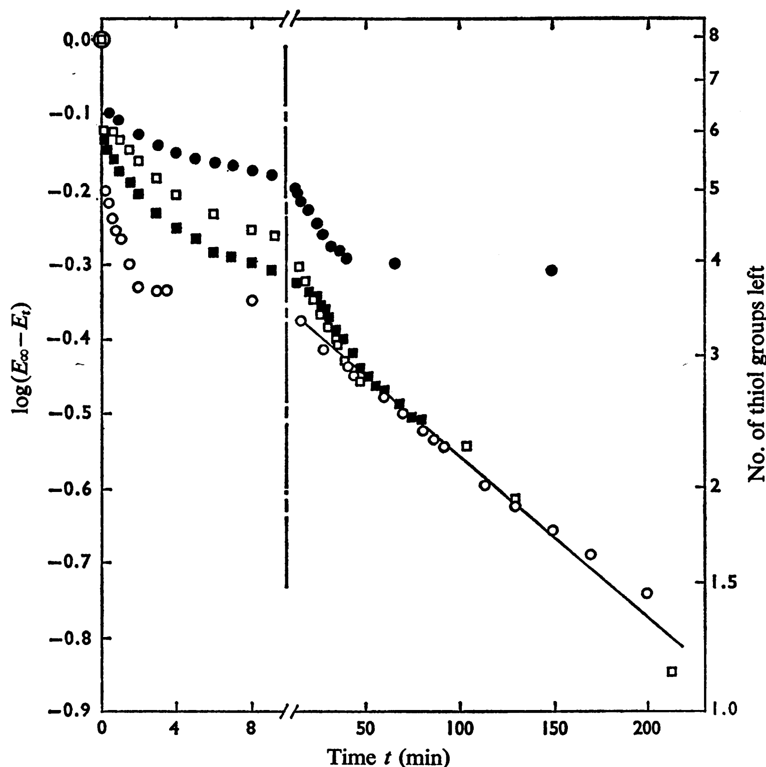

Fig. 4. *Reaction of acetyl-CoA synthetase with p-hydroxymercuribenzoate at* 20.5°C

Reaction mixtures (1 ml) contained 0.1 M-Tris–HCl buffer, pH 8.6, 0.2 M-KCl and 111 μg of acetyl-CoA synthetase (15.7 μM enzyme thiol groups). Reactions were initiated by the addition of 10 μl of mercurial to final concentrations of 8.93 μM (●), 17.9 μM (□), 39.3 μM (■) and 78.6 μM (○), and the E_{255} was measured as described in the Experimental Procedures section. $\log(E_{\infty}-E_t)$ at zero time is arbitrarily set equal to zero in all cases. E_{∞} was calculated from the amount of protein added, assuming 8 mol of thiol/57000 g and ϵ of 6.38 litre·mmol^{-1}·cm^{-1}. The straight line in the right-hand portion of the figure is drawn for the equation $2.3\log(E_{\infty}-E_t) = kt$, with a first-order constant, k, of 5×10^{-3} min^{-1}.

of 1 mM-mercaptoethanol, the very rapid inhibition described above did not occur. Instead, an exponential loss of activity was observed (Fig. 7). The first-order rate constant for this decay was independent of the mercurial concentration and of the presence of mercaptoethanol in the incubation mixtures, provided that the mercurial was in excess of the total thiol (Fig. 7). With mercurial concentrations lower than that of mercaptoethanol, inactivation still occurred, but at a much smaller rate. In the absence of mercaptoethanol and with sufficient mercurial to react with only 2.8 enzyme thiol residues, the irreversible inactivation, although slow, proceeded eventually to at least 90% of completion. Under the conditions used in the experiments of Fig. 7, the loss of enzymic activity in the absence of the mercurial was less than 10%/24 h.

This irreversible inactivation could be easily studied with enzyme of high specific activity (40 units/mg) from which the mercaptoethanol had not been removed, because the constant rate observed at high mercurial concentrations permitted the use of excess of *p*-hydroxymercuribenzoate (as a routine 100–200 μM). This slow process displayed the same energy of activation (+136 kJ/mol) as did the reaction of the last four thiol residues with the mercurial (Fig. 8). The rate of inactivation was proportional to the enzyme concentration between 12 and 170 μg/ml, and did not change between *I* 0.2 and *I* 0.44, but was 40% faster at *I* 0.04. Replacement of 0.2 M-KCl by 0.2 M-NaCl did not alter the rate. Between pH 7.0 and 8.3 the rate was independent of pH; at pH 6.2 it was about 10% less, and at pH 8.9 about 10% greater.

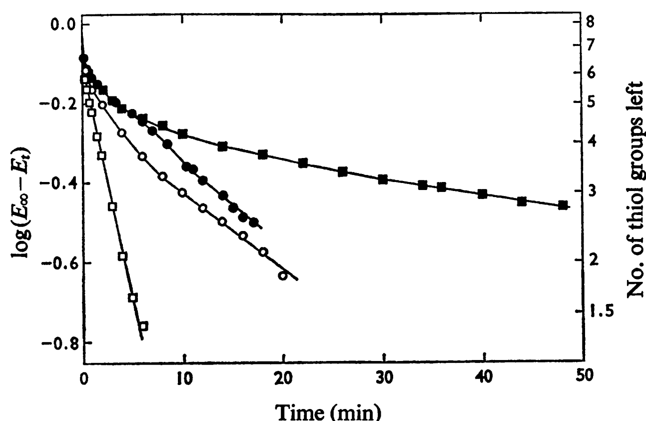

Fig. 5. *Effect of temperature and pH on the reaction of acetyl-CoA synthetase with p-hydroxymercuribenzoate*

Enzyme (113 μg/ml, equivalent to 15.9 μM protein thiol) was treated with 20 μM-mercurial under the following conditions: 20.5°C, pH 8.4 (■); 30.5°C, pH 8.4 (○); 30.5°C, pH 7.1 (●). At 39.0°C and pH 8.4, 74 μg of enzyme/ml equivalent to 10.4 μM protein thiol was treated with 20 μM-mercurial (□). Other experimental conditions and the method of calculation were as described in Fig. 4.

Effects of substrates on the rate of irreversible inactivation

Unless otherwise stated the initial rates of inactivation in the presence of substrates were first-order during the loss of at least 60% of the enzymic activity. An apparent first-order constant ($k_{obs.}$) was calculated from this initial exponential phase, and the ratio, $k_{obs.}/k_1$ (where k_1 is the rate constant for the inactivation in the absence of substrate), was used as a measure of 'protection'. In Fig. 9, $k_{obs.}/k_1$ is plotted against the concentration of ATP in various conditions. The protection was only partial; $k_{obs.}/k_1$ approached a limiting value of about 0.14 at high ATP concentrations. At pH 8.3, the concentration of ATP required for half-maximal protection [$K_{\frac{1}{2}}$(ATP)] was less when the concentration of $MgCl_2$ was kept at 5 mM, than when $MgCl_2$ and ATP were present in equal amounts. At 5 mM-$MgCl_2$ [$K_{\frac{1}{2}}$(ATP)] decreased from about 260 μM at pH 8.3, to <30 μM at pH 7.0. In 10 mM-potassium phosphate, pH 7.0, containing 0.2 M-KCl and 10 mM-$MgCl_2$, [$K_{\frac{1}{2}}$(ATP)] was somewhat larger (not shown) than in the Tris buffer system at the same pH value, but in phosphate buffer at pH 6.2, [$K_{\frac{1}{2}}$(ATP)] was approx. 12 μM. ATP did not protect the enzyme in the absence of $MgCl_2$ and presence of 1.0 mM-EDTA in the Tris buffer system at either pH 7.0 or 8.3.

In Table 3 are shown the effects of some other compounds on the rate of inactivation. The protective effect of $MgCl_2$ alone was slight. At pH 7.0, in the presence of 5 mM-$MgCl_2$ there was a small, but definite, additional protection by acetate. AMP protected the enzyme with a half-maximal effect at about 2 mM both at pH 8.3 in the absence of $MgCl_2$ and at pH 7.0 in the presence of 5 mM-$MgCl_2$. Significant protection occurred with 0.9 mM-acetyl-CoA at pH 7.0. Attempts were made to investigate the binding of acetate to the enzyme by using acetate to displace acetyl-CoA from the enzyme. However, instead of the expected antagonistic effect of these two substrates, a much greater protection was observed in the simultaneous presence of 0.9 mM-acetyl-CoA and 6.5 mM-acetate than with either alone, and with 0.1 mM-acetyl-CoA plus 6.5 mM-acetate the plot of logarithm of remaining activity versus time was a smooth curve of decreasing slope.

Although pyrophosphate caused little or no protection, it prevented the protection caused by ATP (Table 3). At pH 7.0, in the presence of 10 mM-acetate and 5 mM-$MgCl_2$, protection of the enzyme by 33 μM-ATP was nearly complete ($k_{obs.}/k_1 = 0.05$). Under these conditions, protection was observed with ATP concentrations comparable with that of the enzyme and attempts were made to titrate the enzyme with ATP. The concentration of ATP required to protect the enzyme appeared to be proportionately higher at a higher enzyme concentration (Fig. 10). At both enzyme concentrations, the progress curves

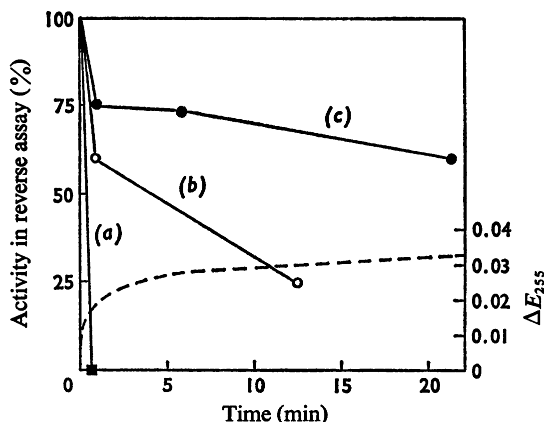

Fig. 6. *Reversible inhibition of acetyl-CoA synthetase by p-hydroxymercuribenzoate*

Enzyme was incubated with mercurial at 31.5°C in 0.1M-Tris–HCl buffer, pH8.3, containing 0.2M-KCl. At the times indicated after addition of the mercurial, 20μl portions were tested for activity in the reverse reaction assay as described in the Experimental Procedures section (——). In experiment (*a*), 173μg of enzyme/ml (24.3μM protein thiol) in the presence of 288μM-mercaptoethanol was treated with 438μM-mercurial (■). In experiments (*b*) and (*c*) enzyme at 78μg/ml and 156μg/ml respectively, i.e. 11.0 and 21.9μM protein thiol, was treated with 5.54μM-mercurial, equivalent to 4 and 2 of the total enzyme thiol residues. ---- shows the increase in E_{255} accompanying mercaptide formation under conditions identical with those of experiment (*b*).

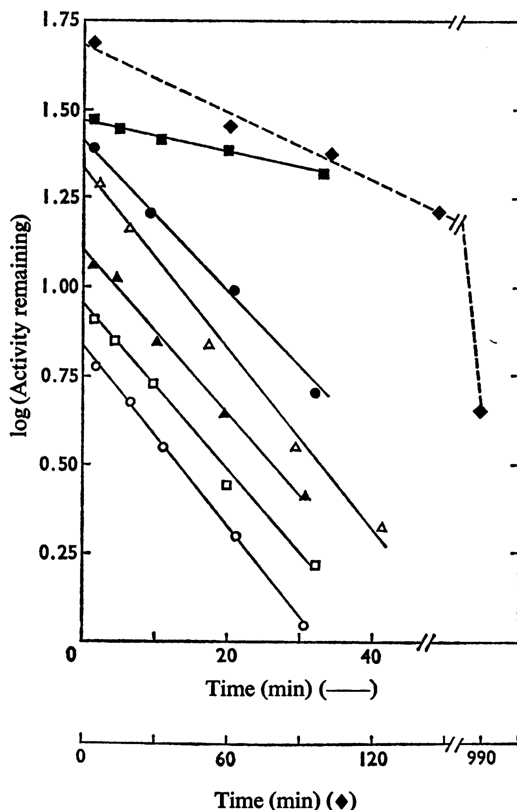

Fig. 7. *Irreversible inactivation of acetyl-CoA synthetase by p-hydroxymercuribenzoate in* 0.1M-*Tris–HCl buffer, pH*8.3, *containing* 0.2M-*KCl at* 31.4°C

In one experiment (●), 173μg of enzyme/ml, equivalent to 24.3μM protein thiol, was incubated with 438μM-mercurial in the presence of 288μM-mercaptoethanol. At the times indicated, 20μl portions were tested for activity in the reverse reaction assay as described in the Experimental Procedures section with assay mixtures containing 1mM-mercaptoethanol. In the remaining experiments, activity decay was followed by testing portions in the standard coupled assay as described in the Experimental Procedures section. Conditions were as follows: in the presence of 110μM-mercaptoethanol, 26μg of enzyme/ml (3.7μM protein thiol) was incubated with mercurial at total concentrations of 508μM (○), 302μM (□), 151μM (△) and 76μM (■). In the absence of mercaptoethanol, 12μg of enzyme/ml (1.7μM protein thiol) was incubated with 151μM-mercurial (▲), and 75μg of enzyme/ml (10.5μM protein thiol) with 3.75μM-mercurial, equivalent to 2.8 of the 8 thiol residues (◆). Note the different time-scale for the last experiment.

for the inactivation showed unusual features, which are not understood; at the higher ATP concentrations (above 30μM), a slight increase in activity (about 8%) was observed during the first 10min after addition of *p*-hydroxymercuribenzoate, and at lower ATP concentrations (<15μM) a sharp acceleration in the decay was seen after 40min of exponential inactivation. These features did not change when the enzyme was preincubated with $MgCl_2$, acetate and ATP for 20min (instead of the usual 1min) before addition of the mercurial. In all cases, the rate constant of the exponential phase of inactivation was used in Fig. 10.

Discussion

Acetyl-CoA synthetase purified by the procedure described here is much more stable and of 70% higher specific activity than that obtained by earlier methods (Webster, 1965, 1969). The molecular weight of the enzyme eluted from TEAE-cellulose is constant over

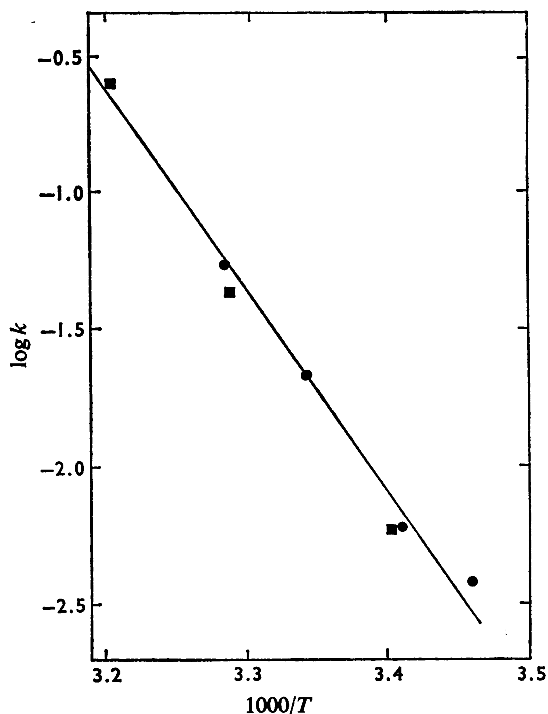

Fig. 8. *Effect of temperature on the irreversible inactivation of acetyl-CoA synthetase and on the rate of reaction with p-hydroxymercuribenzoate of the last 4 thiol residues*

The logarithms of the first-order constants (k) (expressed in min^{-1}) for enzymic inactivation (●) and for the rate of mercaptide formation from the last 4 thiol residues in the protein (■) are plotted against the reciprocal temperatures. Rates of decay of activity were determined by measuring activity in the standard coupled assay of portions of incubation mixtures containing a large excess of mercurial (~100 μM) as explained in Fig. 7. Rates of mercaptide formation were calculated from the data of Figs. 4 and 5.

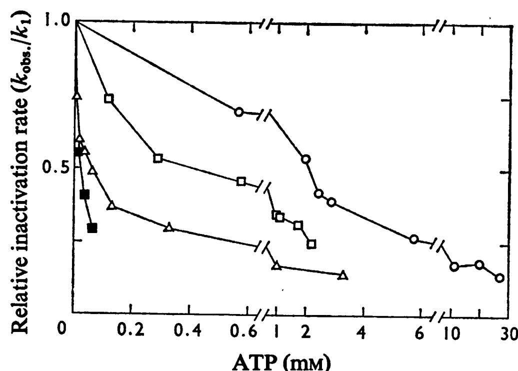

Fig. 9. *Protection of the enzyme by ATP against the irreversible inactivation by p-hydroxymercuribenzoate at* 31.4°C

The relative rate of inactivation, $k_{obs.}/k_1$ (where k_1 is the rate in the absence of ATP), is plotted against the ATP concentration for experiments (*a*) in 0.1 M-Tris–HCl–0.2 M-KCl, pH 8.3, with $MgCl_2$ at 5 mM (□), (*b*) in 0.1 M-Tris–HCl–0.2 M-KCl, pH 8.3, with $MgCl_2$ equal to the ATP concentration (○), (*c*) in 0.1 M-Tris–HCl–0.2 M-KCl, pH 7.0, with $MgCl_2$ at 5 mM (△), and (*d*) in 10 mM-potassium phosphate–0.2 M-KCl, pH 6.22, at 10 mM-$MgCl_2$ (■).

a 1000-fold range of protein concentrations and is about twice that previously found by sedimentation-equilibrium measurements near the meniscus with aggregating crystalline enzyme (Webster, 1965). The identical gel-filtration behaviour of the enzyme in the presence of 130 mM-KCl, -NaCl or -tetramethylammonium chloride shows that the mechanism of activation by K^+ ions and inhibitions by Na^+ ions (see, e.g., Webster, 1966) does not involve any change in molecular weight.

The molecular weight (57000) obtained by analytical gel filtration is at best only accurate to ±5%, and may contain a systematic error if the protein behaves abnormally on Sephadex. This must be considered, because preparative gel filtration of enzyme concentrated from TEAE-cellulose eluates caused changes in the sedimentation behaviour and a loss of activity. However, after a second gel-filtration step, sedimentation-equilibrium measurements near the meniscus indicated a $\bar{M}_z$ of 72000, consistent with a limiting molecular weight close to 57000. The high-molecular-weight shoulder in the activity profile of many gel-filtration experiments may represent material already present in the TEAE-cellulose eluates, or material formed during analytical gel filtration.

The amino acid analysis and u.v. absorption indicate that CoA is not present in the purified enzyme.

The kinetic studies of the reaction of the thiol groups of native enzyme with *p*-hydroxymercuribenzoate show two distinct phases (Fig. 4). During dialysis of the enzyme to remove mercaptoethanol, about 15% of the activity was lost, without an obvious decrease in the number of thiol residues. The reactivity of thiol residues in the inactivated enzyme might not have been the same as in native enzyme. However, the results of Figs. 6, 7 and 8 show that in both the main phases of the reaction with the

Table 3. *Effect of substrates on the initial rate of inactivation of acetyl-CoA synthetase by p-hydroxymercuribenzoate at* 31.5°C

Initial first-order rates of irreversible inactivation ($k_{obs.}$) were measured by the standard coupled assay as described in the Experimental Procedures section, and k_1 is the first-order constant for the inactivation in an incubation without added compounds. Control and test incubations were in 0.1 M-Tris–HCl buffer containing 0.2 M-KCl, at the pH and $MgCl_2$ concentration listed. The concentrations of enzyme, total mercurial and mercaptoethanol were about 50 μg/ml, 150 μM and 100 μM respectively, and were kept constant in experiments with the same number, done on the same day.

Expt. no.	Added compound	pH	$MgCl_2$ (mM)	$10^3 k_{obs.}$ (min^{-1})	$k_{obs.}/k_1$
1	$MgCl_2$ (5 mM)	8.3	—	52	0.96
	$MgCl_2$ (10 mM)	7.0	—	53	0.95
	EDTA (5 mM)	7.0	0	53	0.95
2	Acetate (5 mM)	8.3	0	54	0.97
	Acetate (10 mM)	8.3	0	54	0.97
	Acetate (54 mM)	8.3	0	50	0.90
	Acetate (54 mM)	8.3	1.0	55	0.98
3	Acetate (10 mM)	7.0	5.0	42	0.84
4	Acetate (6.5 mM)	7.0	5.0	47	0.81
5	AMP (0.44 mM)	8.3	0	50	0.90
	AMP (1.22 mM)	8.3	0	42	0.75
	AMP (4.44 mM)	8.3	0	24	0.43
4	AMP (0.74 mM)	7.0	5.0	39	0.67
	AMP (2.46 mM)	7.0	5.0	29	0.50
	AMP (11.1 mM)	7.0	5.0	14	0.24
6	PP_i (1.0 mM)	8.3	5.0	45	0.90
	PP_i (4.5 mM)	8.3	5.0	47	0.94
	PP_i (4.5 mM)*	8.3	5.0	50	1.00
	PP_i (2.5 mM)	7.0	2.5	47	0.94
7	ATP (1.14 mM)	8.3	5.0	16	0.35
	ATP (1.14 mM)+PP_i (2.0 mM)	8.3	5.0	31	0.67
3	ATP (33 μM)	7.0	5.0	30	0.56
	ATP (33 μM)+PP_i (3.0 μM)	7.0	5.0	41	0.81
	ATP (33 μM)+PP_i (10.0 μM)	7.0	5.0	45	0.90
	ATP (33 μM)+PP_i (100 μM)	7.0	5.0	48	0.97
8	Acetyl-CoA (0.2 mM)	7.0	5.0	53	0.92
	Acetyl-CoA (0.9 mM)	7.0	5.0	34	0.59
	6.5 mM-Acetate present in test and control in the following experiments:				
8	Acetyl-CoA (0.04 mM)	7.0	5.0	45	0.97
	Acetyl-CoA (0.1 mM)	7.0	5.0	Decay not exponential	
	Acetyl-CoA (0.9 mM)	7.0	5.0	11	0.23

* KCl replaced by NaCl.

mercurial catalytic activity was affected, and therefore residues in the native fraction of the enzyme were being modified. The general picture of thiol reactivity observed therefore applies to native enzyme, although quantitative interpretations must be made with caution. In experiment (*b*) of Fig. 6, direct comparison between the number of thiol residues modified and the reversible inhibition can be made; reaction of 2.1 and 3.4 residues caused respectively 40 and 70% inhibition. With the high mercurial concentration used in experiment (*a*), about 4.2 residues are very rapidly modified (cf. Fig. 4), and 100% inhibition was found within 0.7 min, by which time reaction of the last 4 residues and the

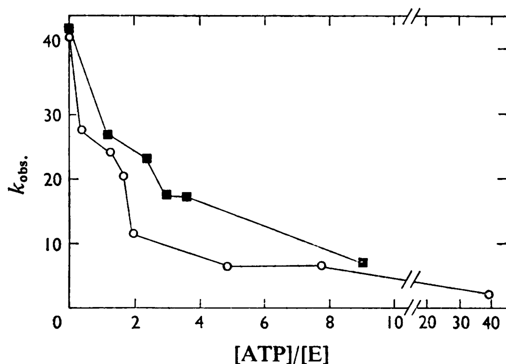

Fig. 10. *Protection by ATP against the irreversible inactivation by p-hydroxymercuribenzoate at* 31.4°C *in* 0.1 M-*Tris–HCl buffer, pH*7.0, *containing* 5 *m*M-*$MgCl_2$ and* 10 *m*M-*potassium acetate*

The observed first-order rates of inactivation for the first 40min ($k_{obs.}$) are plotted against the molar ratio of ATP to enzyme ([ATP]/[E]) at enzyme concentrations of 0.85 μM (○) and 3.42 μM (■).

irreversible inactivation have scarcely started (cf. Figs. 4, 5 and 7). Thus the reversible inhibition lags slightly behind the extent of modification of the first four thiol residues, possibly because of preferential modification of residues in the inactive enzyme present at the beginning of the experiment. This proportionality cannot be shown by a simple (i.e. time-independent) titration of the activity with the mercurial because of interference from the slow irreversible inactivation.

Inhibition of the enzyme by *p*-hydroxymercuribenzoate can be satisfactorily explained without supposing a direct role in the catalysis for any thiol residue. Thus the irreversible inactivation proceeded at the same rate and with the same large energy of activation as the modification of the last four thiol residues, and it is probable that both processes are controlled by a rate-limiting change in protein conformation. A conformation change may also accompany modification of the first four residues, since this is much slower than the reaction of unhindered thiol groups with hydroxymercuribenzoate (e.g. under the conditions of Fig. 4, CoA reacts completely within 0.2min), and a slow irreversible inactivation is initiated after modification of only 2.8 residues (Fig. 7).

These conclusions concur with those of Sharkova (1968). She was able to titrate 8.9 thiol residues/57000g of rabbit heart acetyl-CoA synthetase with *p*-hydroxymercuribenzoate in 2h at 0°C, without inhibition of the partial reaction between acetyl adenylate and pyrophosphate. This does not exclude a role for thiol residues in the overall reaction, as Sharkova (1968) pointed out. The greater availability of the thiol residues in the rabbit enzyme may result from its smaller molecular weight, of about 32000 (Sharkova, 1968).

Further evidence against a direct role in the catalysis for the first four thiol residues of the ox enzyme is that all substrates, except CoA, failed to protect the enzyme against the reversible inhibition although they did alter the rate of irreversible inactivation. They must therefore be able to bind to enzyme in which four thiol residues have been modified. For this reason, the protection studies must be treated with caution, for it is not clear how closely they reflect binding to native enzyme. With this reservation, the following conclusions may be drawn from these protection studies. In the presence of $MgCl_2$, all the substrates, except possibly CoA, are able to form binary complexes with enzyme. Protection by AMP did not require $MgCl_2$, and [$K_{\frac{1}{2}}$(AMP)] (about 2mM) showed little pH dependence between pH 7.0 and 8.3. The absence of a Mg^{2+} requirement is consistent with the Mg^{2+}-independent reaction of acetyl adenylate and CoA to form AMP and acetyl-CoA (Berg, 1956; Webster, 1967). Half-maximal protection by acetyl-CoA occurred at about 1mM. The enhancement by acetate of protection by acetyl-CoA is peculiar, and suggests that acetate and acetyl-CoA can simultaneously bind to the enzyme. At pH 7.0, and in the presence of 5mM-$MgCl_2$, pyrophosphate appears to have a dissociation constant from the enzyme two orders of magnitude smaller than those of AMP and acetyl-CoA. Thus 3 μM-pyrophosphate decreased by 57% the protection afforded by 33 μM-ATP, which itself has a $K_{\frac{1}{2}}$ less than 30 μM under these conditions. Like that of ATP, the binding of pyrophosphate was strongly pH dependent: at pH 8.3 2mM-pyrophosphate was required to decrease by 50% the protection afforded by 1.1mM-ATP.

Protection by ATP required the presence of $MgCl_2$. If ATP can bind to the enzyme in the absence of $MgCl_2$, it does so without affording protection. The increase in [$K_{\frac{1}{2}}$(ATP)] between pH 7.0 and pH 8.3 (Fig. 9) reflects a change in the apparent K_m for ATP from 17 μM at pH 6.7 to 350 μM at pH 8.3 in Tris buffer (J. C. Londesborough, unpublished work). The results shown in Fig. 9 at pH 8.3 with equal $MgCl_2$ and ATP concentrations are consistent with an enzyme–ATP complex with a dissociation constant of 1.2mM. The results obtained at constant $MgCl_2$ concentrations cannot be fitted by a single dissociation constant. Protection at low ATP concentrations was greater than would be predicted from the behaviour at higher concentrations. A more direct method for studying ATP binding is required to distinguish the possible explanations, which include the occurrence of different classes of ATP binding

sites or negative co-operativity, but also a non-linear relation between the rate of inactivation and the number of empty binding sites. Different classes of ATP binding sites may also explain the need for proportionately more ATP at larger enzyme concentrations to 'titrate' the inactivation in the presence of acetate (Fig. 10).

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