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ASSOCIATIVE PROPERTIES OF BUTYRYL-COENZYME A SYNTHETASE FROM OX LIVER MITOCHONDRIA

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Summary

Butyryl-coenzyme A synthetase (butyrate:CoA ligase (AMP-forming), EC 6.2.1.2) from an acetone-dried powder of ox liver mitochondria was found to have a molecular weight of approx. 40 000. The sedimentation equilibrium analysis suggested the presence in solution of higher molecular weight forms of the enzyme and these could also be obtained by extracting the enzyme from the mitochondrial powder in non-reducing conditions. The enzyme was inhibited by sulphydryl reagents, and was found to have at least one available thiol group/molecule. The relationship between enzymic activity and concentration was non-linear, and suggested that an inactive monomer-active dimer equilibrium was present. The 5—6-fold activation by bovine serum albumin required the presence of free thiol groups in the albumin and involved association of albumin with the enzyme.

Introduction

Some conflicting reports have been published on the mechanism of action of the medium chain-length fatty acyl-CoA synthetase, butyryl-CoA synthetase (butyrate:CoA ligase (AMP-forming), EC 6.2.1.2), which catalyses the formation of fatty acyl-CoA from free fatty acids containing 4—12 carbon atoms:

$$RCO_2H + CoA \cdot SH + ATP \rightleftharpoons R \cdot CO \cdot SCoA + AMP + PP_i$$

The acyl-AMP derivative was implicated [1,2] in a mechanism similar to that

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proposed by Berg [3] for acetyl-CoA synthetase. However, using isotope exchange techniques, Jones et al. [4] had earlier suggested an alternative Ping-Pong mechanism, not involving an acyl-AMP intermediate. Further support for this latter mechanism was obtained on the basis of product inhibition studies [5]. Bar-Tana et al. [6] separated the ox liver enzyme into two interconvertible fractions, one apparently catalysing the reaction by an ordered mechanism [7], the other [8] by the Berg mechanism.

As part of an attempt to resolve these differences, the properties of the enzyme were reinvestigated using a more sensitive assay [9] and purer preparation than that used previously [5]. This [10] indicated a much more complex kinetic behaviour of the enzyme than was noted with the cruder fraction and the higher protein and CoA concentrations [5], and the present results suggest the ability of the enzyme to associate both with itself and with other protein molecules.

A preliminary communication of part of this work has been given elsewhere [11].

Materials and Methods

Materials. ATP (98% pure), CoA (85% pure), DL-phosphatidylcholine (synthetic), polyvinylpyrollidone (mol. wt. 10 000), bovine serum albumin (fatty acid-free form and fraction V), azoalbumin, trypsin inhibitor (soybean), ovalbumin, α-chymotrypsinogen, Coomassie brilliant blue G-250, dithiothreitol, iodoacetamide, 5,5′-dithio-bis(2-nitrobenzoic acid), 2-mercaptoethanol, *p*-chloromercuribenzoic acid and cytochrome *c* were purchased from Sigma (London) Chemical Co. (Poole, U.K.). Lactate dehydrogenase (from rabbit muscle) was obtained from the Boehringer Corporation (London) (London, U.K.), acrylamide, *N,N*-methylene-bisacrylamide, $NaBH_4$ and sorbic acid from B.D.H. Chemicals (Poole, U.K.), Sephadex G-200 from Pharmacia (G.B.) (London, U.K.) and Ultrogel AcA34 from LKB Instruments (Selsdon, U.K.). The sorbic acid was purified by recrystallisation from water as the sodium salt and as the free acid using ethanol/water mixtures.

All other reagents were of analytical grade or of the highest purity available. The pH of reagent solutions was adjusted with HCl, KOH or K_2CO_3.

Preparation of enzyme fraction. The enzyme was extracted from acetone-dried ox liver mitochondria [12]. The powder was treated with 100 mM Tris-HCl buffer (pH 8.0), 50 mM mercaptoethanol. After centrifugation at 23 000 × *g* for 30 min to remove debris, the extract was applied to a DEAE-cellulose column (2.2 × 48 cm) previously equilibrated with 20 mM Tris-HCl (pH 8.0), 20 mM mercaptoethanol. The column was then washed with the same buffer until no further protein appeared in the eluate, followed by application of a 0—0.5 M KCl gradient in the same buffer. The enzyme activity eluted as a single sharp peak between 0.1 and 0.2 M KCl.

After concentration of the pooled active fractions using the Amicon ultrafiltration apparatus, the sample (200 mg protein) was put on a Sephadex G-200 or Ultrogel AcA 34 column (2.6 × 58 cm) equilibrated with 100 mM Tris-HCl (pH 8.0), 20 mM mercaptoethanol. The protein was eluted with the same buffer (yield, 54 mg). All steps in the purification procedure were carried out

at 0—4°C. The active fraction was stored at 0°C under N_2.

Assay of butyryl-CoA synthetase. Enzyme activity was determined at 25°C using a direct reading assay described previously [9]. The reaction medium contained (unless otherwise stated): 50 mM Tris-HCl (pH 8.0), 10 mM mercaptoethanol, 0.04 mM CoA, 10 mM $MgCl_2$, 10 mM ATP, 1 mM sorbate, and 20—500 μg enzyme protein in a final volume of 1 ml. Absorbance changes were measured for 2—6 min at 300 nm in a 1 cm cell using a Pye Unicam SP1800 spectrophotometer, and the activity calculated from the gradient of the linear portion of the absorbance against time, assuming a value of 19 300 $l \cdot mol^{-1} \cdot cm^{-1}$ for the molar extinction coefficient of sorboyl-CoA [13].

Polyacrylamide gel electrophoresis. This was carried out at 1—4°C essentially as described by Tombs and Akroyd [14], using 7.5% gels and discontinuous buffer system; gel buffer 400 mM Tris-HCl (pH 8.9), reservoir buffer 40 mM glycine titrated to pH 8.0 with concentrated Tris. Protein samples were stained with Coomassie brilliant blue G-250 [15]. To detect activity, the gels were halved longitudinally, one-half stained, and the other half sliced transversely at sections corresponding to the protein bands. The sections were macerated in a test tube with a small volume of 50 mM Tris-HCl (pH 8.0), 20 mM mercaptoethanol and the activity assayed after 1 h at 0°C. 100 μg bovine serum albumin was included in the assay mixture to increase sensitivity.

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate was carried out as described by Weber and Osborn [16] using 7.5% gels but with 0.4 M Tris-HCl (pH 8.0) in the gel and same buffer diluted to 0.08 M in the reservoir. The standard marker proteins used with their corresponding molecular weights were: bovine serum albumin (67 000), ovalbumin (45 000), α-chymotrypsinogen (25 000), and soybean trypsin inhibitor (21 600).

Ultracentrifugal analysis. Sedimentation equilibrium experiments were performed with a Spinco Model E analytical ultracentrifuge using both schlieren and interference optics [17]. Attainment of equilibrium (attained well within 24 h) was checked from the interference patterns. The molecular weight was calculated from the schlieren pattern using a weighted fit to the log schlieren plot [18]. The run was carried out under both meniscus depletion (29 500 rev./min) and conventional (17 980 rev./min) sedimentation conditions at 8°C with a 1.5 mg/ml enzyme fraction in 83.4 mM Tris-HCl (pH 8.0), 0.16 mM dithiothreitol, 1.67 mM *p*-chloromercuribenzoate. In calculating molecular weights, a value of 0.725 $cm^3 \cdot g^{-1}$ was assumed for the partial specific volume of the protein.

Molecular weight determinations by gel permeation chromatography. These were carried out by the method of Andrews [19] at 1—4°C using an Ultrogel AcA34 column (2.6 × 58 cm). Elution volumes (V_e) are expressed as the ratios to the dextran blue exclusion volume (V_0). The standard proteins were those used in electrophoresis. The eluting buffer was normally 100 mM Tris-HCl, 100 mM KCl, 25 mM mercaptoethanol (pH 7.8).

Assay of protein and CoA. These were determined by the biuret-phenol [20] and phosphotransacetylase [21] methods, with bovine serum albumin as standard in the former case. Where high concentrations of mercaptoethanol interfered with the protein assay, the solution was dialysed against the same buffer without mercaptoethanol to remove the interfering substance.

Iodoacetamide derivative of bovine serum albumin. This was prepared as described by Waxdal et al. [22], but without denaturation of the albumin with guanidine hydrochloride.

Results

Properties of the final active fraction

The enzyme showed lability towards air oxidation, particularly in the purified state. In preparations which had become oxidised, the sample was irreversibly aggregated and eventually precipitated. The precipitate could not be redissolved or reactivated by reducing agents such as dithiothreitol, mercaptoethanol, or $NaBH_4$. To attempt to counteract this inactivation, mercaptoethanol (20—50 mM which did not interfere with ultraviolet monitoring) was included in buffers, and the active fraction was stored in ice. Even under these conditions, after about 1 week, a significant amount of aggregation to high molecular weight forms had occurred, as determined by gel permeation chromatography.

The enzyme fraction appeared to be homogenous in molecular weight during ultracentrifugation with the exception of a small amount of rapidly sedimenting material, amounting to less than 5% of the total on the basis of peak areas of the schlieren pattern. The s value of this peak corresponded to a molecular weight of 200 000—300 000. It seems possible that this impurity is the aggregated form.

Polyacrylamide gel electrophoresis of the enzyme resulted in a diffuse broad protein band, in contrast to the sharp bands obtained with standard proteins run at the same time. Enzyme activity was spread throughout this broad band. The presence of substrates gave no significant sharpening of the bands.

Another anomalous property of this enzyme was its specific activity: on this basis, the apparent degree of purification was only three-fold. For example, in one purification, the initital extract of mitochondrial acetone powder contained 2.5 g protein (specific activity 2.4 $mU \cdot mg^{-1}$); after the final purification stage, the yield of 54 mg protein had a specific activity of 6.7 $mU \cdot mg^{-1}$.

Molecular weight determination

The molecular weight of the enzyme was determined by gel permeation chromatography, polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate and sedimentation equilibrium (observed values, 40 000, 38 000 and 41 000 ± 1000, respectively). This value of about 40 000 was also confirmed during several purifications of the enzyme by gel chromatography, not only with the fraction obtained from DEAE-cellulose, but also in an earlier experiment with a sample obtained by $(NH_4)_2SO_4$ fractionation of the crude extract. SDS gel electrophoresis gave only a single band, suggesting that the protein was at least largely homogeneous in molecular weight. The ultracentrifugation was carried out under meniscus depletion conditions: the sample at the lower speeds of 'conventional' sedimentation equilibrium gave a plot of $-\ln[(1/r) \cdot (dc/dr)]$ versus r^2 which curved to indicate the presence of higher molecular weight forms, and this suggests aggregation of the enzyme. The centrifugation was also in the presence of *p*-mercuribenzoate to discourage

aggregation by the formation of disulphide linkages: this, however, did not affect the tendency of the enzyme to aggregate.

Inactivation of the enzyme by sulphydryl reagents

The enzyme was readily inactivated by *p*-chloromercuribenzoate, 5,5'-dithiobis(2-nitrobenzoic acid) and iodoacetamide.

p-Mercuribenzoate. The ready oxidation of the enzyme suggested some advantage in isolating it as the *p*-mercuribenzoate derivative, and for this reason the reversal of the inhibition of the enzyme was examined further. In the presence of substrates at the assay mixture concentrations, but with 0.5 mM mercaptoethanol (instead of 10 mM), activity was completely abolished by 1 mM *p*-mercuribenzoate. On adding an excess of mercaptoethanol after 10 min, the activity returned to its initial value. In the absence of substrates, however, an irreversible inactivation was found to take place (Fig. 1). The reaction followed first-order kinetics with apparent rate constant 0.041 min^{-1}. With substrates present at the concentrations used in the assay, the results tended to be more complex with even an apparent activation in the first 5—10 min in the presence of either MgATP or CoA. With CoA or sorbate there was little evidence of any long-term stabilization of the enzyme, all activity being lost within 55 min, though this did appear to be the case with MgATP (Fig. 1). However, even in the latter case all activity was lost on leaving the enzyme solution containing inhibitor and ATP overnight at 0°C.

Iodoacetamide. The enzyme was also irreversibly inhibited by 2.5 mM iodoacetamide. After dialysis against 100 mM Tris-HCl (pH 8.0) the inactive derivative was unstable and precipitated in 1—2 days at 0—4°C.

5,5'-Dithiobis(2-nitrobenzoic acid). In the absence of mercaptoethanol but with all other reagents of the assay mixture present, the enzyme treated with an excess of this inhibitor was inactive. On adding mercaptoethanol to a concentration of 10 mM, an increase in activity was observed, reaching a maximum

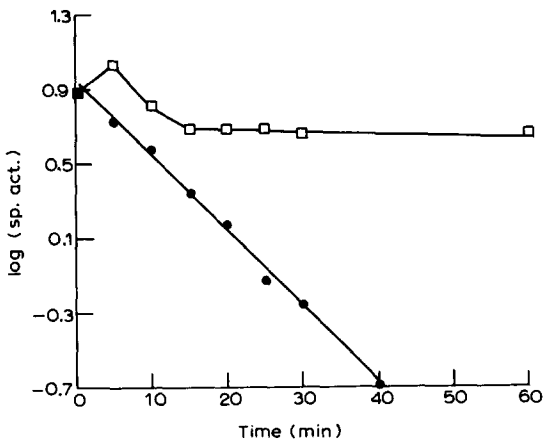

Fig. 1. Enzyme inactivation by *p*-chloromercuribenzoate. 0.54 mg enzyme protein in a total volume of 1 ml containing 50 mM Tris-HCl (pH 8.0), 0.1 mM dithiothreitol and 3 mM *p*-chloromercuribenzoate was incubated at 0°C in the presence (□) and absence (●) of 10 mM MgATP. 100-µl samples were removed at intervals for analysis of activity.

after 20 min at 25°C. However, in a later experiment in which an attempt was made to isolate the inhibited derivative of the enzyme by gel permeation chromatography on Ultrogel AcA34, the protein precipitated on the column.

To determine the number of thiol groups present, the enzyme solution was dialysed at 0—4°C against 50 mM Tris-HCl (pH 8.0), 10 mM mercaptoethanol, 5 mM KBH_4 overnight, then exhaustively against 50 mM potassium phosphate, 20 mM EDTA (pH 7.0) under N_2. 2.8 ml (1.4 mg protein) were then treated with 0.2 ml 10 mM inhibitor, and the change in absorbance at 412 nm measured at 25°C until constant [23]. The value observed, 0.165, corresponds to 1.04 mol thiol groups/40 000 g protein, suggesting the presence of at least one available thiol group/molecule.

A similar result (1.01 mol thiol groups/40 000 g protein) was obtained by reducing the enzyme with 1 mM dithiothreitol, treating it with inhibitor and dialysing out the excess reagents at 0—4°C, and adding to the resulting derivative of the enzyme excess mercaptoethanol; the change in absorbance was measured as before.

Variation in enzymic activity with concentration

The enzyme did not exhibit the expected linear relation between activity and enzyme concentration; instead, there was a distinct lag before activity started to increase with protein concentration (Fig. 2). It is unlikely that this lag arises from protein denaturation in view of the relatively high protein concentrations present (up to 20 $\mu g \cdot ml^{-1}$) when activity was negligible: there is no evidence that this enzyme is particularly sensitive in respect of surface denaturation. Also, the phenomenon was noted even when a silicone-treated cuvette was used.

The activity at concentrations up to 100 $\mu g \cdot ml^{-1}$ is directly proportional to the square of the protein concentration, suggesting that a monomer-dimer equilibrium may be involved with only the dimer enzymically active.

On the assumption that an inactive monomer-active dimer equilibrium is present, with the activity proportional to the concentration of the active

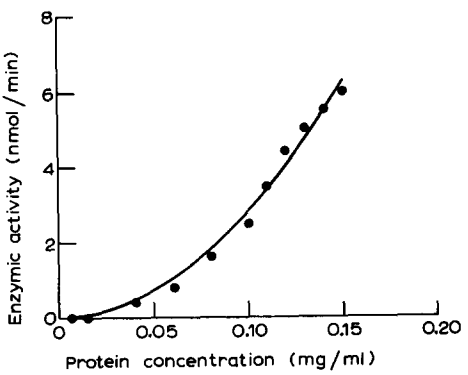

Fig. 2. Variation in activity with concentration of enzyme protein. The protein concentration was that present in the assay mixture. The line represents the fit of the data to the pair of equations: $0.00877E^2 + E - x = 0$, and $y = 280.4\ E^2$, where x and y represent the abscissa and ordinate values.

species, the total enzyme concentration (e) is described by

$$e = E + 2E_2$$

where E and E_2 are the concentrations of monomer and dimer

$$e = E + \frac{2E^2}{K} \tag{1}$$

where $K = E^2/E_2$.

Also the enzymic velocity (v) is related to the concentration of active species by a constant of proportionality, k,

$$v = k \cdot E_2 = \frac{k}{K} \cdot E^2 \tag{2}$$

The data were therefore fitted to Eqns. 1 and 2 using a Fortran program based on the procedure described by Atkins [24], calculations being carried out on the Burroughs B5700 computer system of this University. To allow the inclusion of higher terms in Eqn. 1, an iterative procedure was used for its solution, the model being extended to include both active and inactive trimers and tetramers.

The line in Fig. 2 represents the fit to the basic inactive monomer-active dimer model. The residual variance was not improved by including terms representing active or inactive trimers or tetramers, though a better fit might be obtained if higher molecular weight forms were included. However, these results suggest that at least part of the explanation for this non-linearity arises from the high concentration of inactive monomer present with the active dimer at low enzyme concentrations.

The non-linearity noted in Fig. 2 will, of course, render specific enzyme activity invalid as a criterion of purity.

Activation by bovine serum albumin

The enzyme activity was activated up to six-fold by bovine serum albumin (for example, Fig. 3), but not by a number of other proteins and peptides, including γ-globulin, ovalbumin, azoalbumin and insulin [11]. The enzyme preparation used in Fig. 3 was prepared by an earlier procedure [11], and was not characterised as regards molecular weight. Therefore, the possibility that its molecular weight was other rather than 40 000 cannot be excluded. However, this activation by albumin was a phenomenon noted with all preparations of enzyme in the 40 000 molecular weight form. This activating effect was also noted with fatty acid-free albumin, but not with the derivative of bovine serum albumin obtained by the reaction of the thiol groups of albumin with iodoacetamide. For example, in one experiment in which the activity of 30 μg of enzyme protein/ml of assay mixture was monitored, the measured activity was 0.54 nmol/min. In the presence of 125 μg of the iodoacetamide derivative of bovine serum albumin, the activity showed a slight drop to 0.52 nmol/min. This compared with the 5—6-fold activation consistently observed under similar conditions with bovine serum albumin (e.g. Fig. 3).

To determine whether association of albumin with the enzyme was involved in this activation, mixtures of enzyme and albumin were subjected to gel

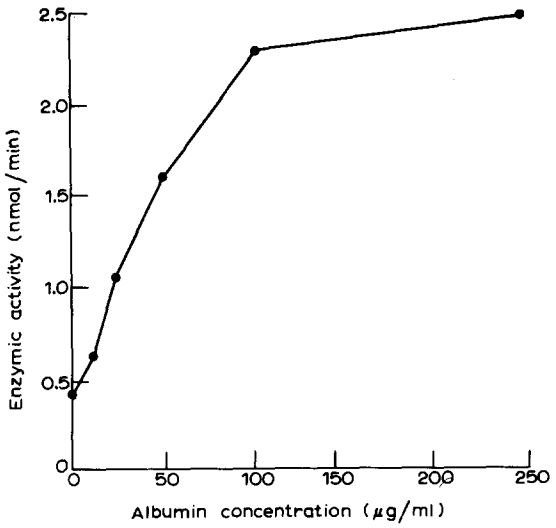

Fig. 3. Enzyme activation by bovine serum albumin. The reaction cuvette contained 0.4 mM $MgCl_2$, 0.4 mM ATP, 1 mM sorbate, 0.04 mM CoA and 76 $\mu g \cdot ml^{-1}$ enzyme protein, along with the indicated amounts of albumin in a total volume of 1 ml.

permeation chromatography. Whereas when treated in this way separately only single protein peaks eluted at the expected V_e/V_0 values, a much more complex pattern is obtained when they are chromatographed together (Fig. 4). Although the main activity peak, D, elutes at a volume corresponding to a molecular weight of approximately 40 000, three other activity peaks appear at lower elu-

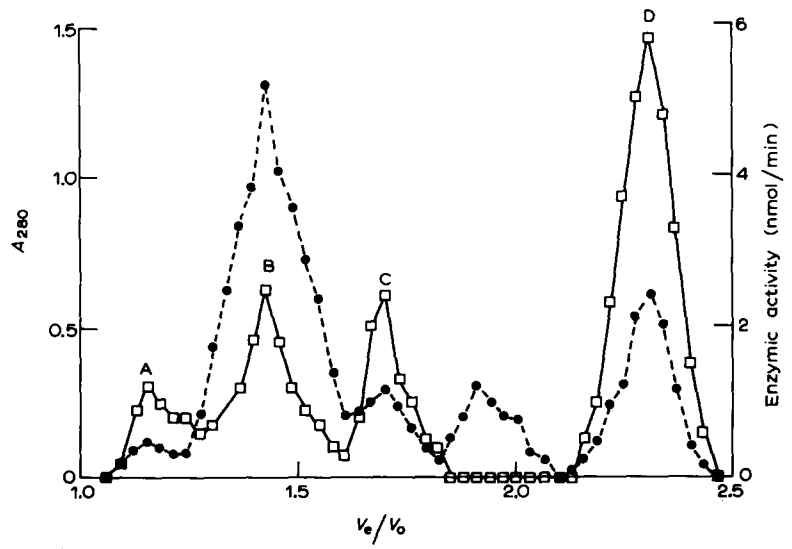

Fig. 4. Gel permeation chromatography on Ultrogel AcA34 of a mixture of enzyme and bovine serum albumin. Enzyme (12 mg) and bovine serum albumin (fatty acid free) (20 mg) in a total volume of 3 ml were eluted with 100 mM Tris-HCl, 10 mM mercaptoethanol (pH 8.2). •, absorbance at 280 nm; □, enzymic activity. Activity peak D corresponds to a mol. wt. of 42 000.

tion volumes, indicating interaction between albumin and enzyme. The elution volumes of these other peaks would only be related to the molecular weights of complexes if they were not in rapid equilibrium with their surroundings. In this particular experiment, extrapolation of the calibration line suggests apparent molecular weights for activity peaks B and C of 174 000 and 112 000. In another similar experiment carried out in the absence of mercaptoethanol, in which enzyme (1.8 mg) and albumin (10 mg) were similarly treated, and using an Ultrogel column which had been calibrated also with lactate dehydrogenase (molecular weight 140 000), a qualitatively similar pattern was observed with activity peaks at elution volumes corresponding to molecular weights of 174 000 and 102 000. The reproducibility of these values suggests that complex formation may not be readily reversible, and that these molecular weights may represent actual species, particularly as these values correspond to those expected for 1 : 1 (theoretical mol. wt. 107 000) and 1 : 2 (theoretical mol. wt. 174 000) enzyme-albumin complexes.

On repeating this experiment with iodoacetamide-treated albumin a very different pattern was noted with only two peaks of activity: one eluting close to the column void volume, and the main one at a volume corresponding to a molecular weight of 58 000. This indicates that association had occurred in this case also, but it is unlikely that this value corresponds to any molecular species, i.e in this case the equilibrium is freely reversible.

The activation by albumin therefore requires both free thiol groups and association with the enzyme.

With an earlier preparation of this enzyme [11], micellar phosphatidylcholine (1.0 mM) [25] and polyvinylpyrrolidone, of mean molecular weights 10 000 (1 mg · ml^{-1}) and 7 · 10^6 (100 $\mu g \cdot ml^{-1}$), appeared to produce slight activations (40%, 9% and 9%, respectively). At higher concentrations the apparent activation by micellar phosphatidylcholine was reduced, and polyvinylpyrrolidone (mol. wt. 7 · 10^6) acted as an inhibitor. In none of these cases was the activation as dramatic as with bovine serum albumin.

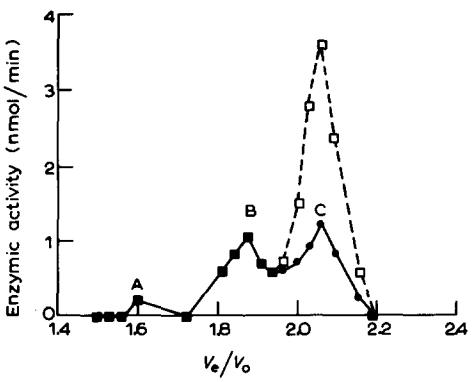

Fig. 5. Gel permeation chromatography on Ultrogel AcA34 of an extract of acetone-dried mitochondrial powder in 100 mM potassium phosphate buffer (pH 7.6). Mitochondrial powder (1 g) was extracted at 2°C for 2 h with phosphate buffer (20 ml), centrifuged to remove debris, concentrated to 4 ml by ultrafiltration and chromatographed. The activity of fractions in the absence of added albumin (•) and in its presence (□) at a concentration of 100 μg/ml in the assay medium was measured.

Extraction of enzymic activity from acetone-dried ox liver mitochondria in non-reducing conditions

As mentioned earlier, the enzyme was very labile towards air oxidation. It was also found that chromatography of a purified enzyme fraction on Ultrogel AcA 34 in the absence of mercaptoethanol produced three activity peaks, corresponding to the 40 000 molecular weight monomer, an apparent tetramer, and a further form of molecular weight of the order of 250 000.

In previous studies [5—8,11], the enzyme has been extracted from acetone-dried ox liver mitochondria by the procedure of Mahler et al. [12], in which the extraction was carried out in the absence of reducing agent. This procedure was therefore investigated to determine whether or not a single homogeneous form was extracted under these conditions. The results shown in Fig. 5 show activity spread over two main peaks corresponding to molecular weights of 80 000 (B) and 40 000 (C), suggesting the presence of both monomer and dimer. Only the 40 000 molecular weight peak was activated by albumin. Extraction in the presence of mercaptoethanol (20 mM) gave rise to a single peak only of molecular weight 40 000.

Discussion

The molecular weight found in this work (40 000) is at variance with earlier values of 47 000 [26], obtained with the enzyme from rat liver mitochondria, and 65 000 [11]. Current work using either ox liver mitochondria or a freeze-dried powder of them has resulted in the isolation of higher molecular weight forms of the enzyme: by extraction of the freeze-dried powder in the presence of mercaptoethanol a 53 000 molecular weight form could be isolated, and treatment of this with ultrasound gave an approximately 45 000 molecular weight form (Ref. 27; Johnston, R.W. and Park, M.V., unpublished data). The latter value is compatible with that observed by Groot et al. [26]. The enzyme appears to be part of a complex. the 40 000 molecular weight unit being liberated by the acetone treatment of the mitochondria.

A time-dependent irreversible inactivation by *p*-mercuribenzoate similar to that found with this enzyme has been reported by Londesborough et al. [28] using acetyl-CoA synthetase from ox heart mitochondria. Their evidence also suggested that a conformational change in the enzyme was the rate-limiting stage in its irreversible inactivation. A similar situation seems possible with the medium-chain acyl-CoA synthetase, the stabilisation by ATP either resulting from the protection of a thiol group, or the holding of the enzyme in an active conformation.

The data in Fig. 2 suggest that under reducing conditions the enzyme is in a reversible monomer-dimer equilibrium with possibly higher molecular weight forms present. These results are analogous to those obtained by Duncan et al. [29] with a bacterial deoxycytidylate deaminase, an inactive monomer-active dimer model also being proposed. This model would explain the diffuse pattern obtained on polyacrylamide gel electrophoresis; like the results of Fig. 2, electrophoresis also indicates that the equilibrium is not significantly affected by the presence of substrates. On oxidation by air, the polymerisation process becomes irreversible, with aggregation and eventually precipitation resulting.

The presence of dimers and tetramers of the 65 000 molecular weight form noted previously during thin-layer gel chromatography [11] even in 10 mM mercaptoethanol presumably results from the oxidation by air of the enzyme and mercaptoethanol present in the thin layer.

Polymerisation on air oxidation may provide an explanation for the two active enzymic fractions obtained by Bar-Tana et al. [6] on ion-exchange chromatography of a fraction from mitochondrial acetone powder. Their eluting buffer contained only 0.04 mM mercaptoethanol, and since during the purification a degree of thiol oxidation by air is inevitable, it is likely under these conditions that some oxidation and aggregation of the enzyme would occur. The authors also comment that reducing conditions seemed to influence the relative proportions of the two enzyme fractions, increasing the relative amount of their fraction I.

The inactive monomer-active dimer model of the enzyme provides a mechanism to explain the activation by albumin. Since albumin associates with the enzyme and since free thiol groups on the albumin are necessary for the activation it appears likely that association of the enzyme monomer with albumin, involving the formation of disulphide bonds, results in an activation analogous to that obtained on dimer formation. The presence of albumin will therefore shift the equilibrium from monomer towards activated forms. The lack of activation of the dimeric form of the enzyme by albumin noted in Fig. 5 agrees with this mechanism. While this activation was noted with albumin alone, it is likely that other proteins may possess this activating ability, including some of those present in the crude fraction. This would explain the poor increase in specific activity noted during the preparation of the enzyme fraction.

Evidence of association of bovine serum albumin with other enzyme molecules has been reported previously by Bernfield et al. [30]. These authors suggested that the ability of bovine serum albumin to reverse the loss of enzymic activity observed at high dilutions of aldolase and lactate dehydrogenase results from complexing of the albumin with the oligomeric forms of these enzymes.

One surprising observation noted here is the apparent formation of associated forms of the enzyme linked by disulphide bonds in the presence of, for example, 10 mM mercaptoethanol (Fig. 4). It suggests a very high affinity of one or more of the enzyme thiol groups for disulphide bond formation, possibly resulting from stabilisation of the disulphide dimer by other associative forces between the two protein molecules, on analogy with the favourable free energy change when dithiothreitol is oxidised to the sterically favoured cyclic disulphide [31].

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