Rat Liver Microsomal Palmitoyl-Coenzyme A Synthetase

STRUCTURAL PROPERTIES

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The structural properties of purified palmitoyl-CoA synthetase from rat liver microsomal material were investigated. The active enzyme has a molecular weight of 168000 and contains about 8.2 mol of phospholipid bound/mol of enzyme protein, as well as bound fatty acids. Association of the active enzyme was shown to occur, without impairment of catalytic activity. The lowest-molecular-weight species obtained under denaturing conditions was 27000 daltons.

Rat liver microsomal long-chain fatty acyl-CoA synthetase (EC 6.2.1.3) catalyses the biosynthesis of the appropriate acyl-CoA ester in the presence of ATP, CoA and the respective fatty acid (Kornberg & Pricer, 1953). Since this enzyme catalyses the first metabolic step leading to either oxidation or incorporation into lipids of long-chain fatty acids it has a key role in lipid as well as energy metabolism. The enzyme was isolated by deoxycholate solubilization of a partially delipidated rat liver microsomal fraction followed by fractionation by means of ion-exchange, adsorption and gel-filtration techniques (Bar-Tana *et al.*, 1971). The purified enzyme fraction catalysed the overall reaction by a Bi Uni Uni Bi Ping Pong mechanism, where ATP and fatty acid first combine with the enzyme to yield an enzyme-bound intermediate, which subsequently reacts with CoA to yield the final acyl-CoA product (Bar-Tana *et al.*, 1973). The intermediate concerned was isolated and shown to consist of fatty acid and AMP bound to enzyme in a molar ratio of 1:1 (J. Bar-Tana, G. Rose & B. Shapiro, unpublished work).

Characterization of the structural properties of the enzyme protein is of interest because of its tight binding to the microsomal membrane and its involvement in lipid metabolism. The structure may reflect protein-protein as well as protein-lipid interactions related to membrane assembly of catalytic units.

Experimental

Materials

Guanidinium chloride was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and was crystallized as described by Nozaki & Tanford (1967). Sodium dodecyl sulphate was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sephadex G-50 (fine grade) and Sepharose

6-B were from Pharmacia, Uppsala, Sweden and ampholites for electrofocusing were from LKB Produkter AB, Bromma, Sweden. Protein markers in the molecular-weight studies were obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Lactate dehydrogenase was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. All other chemicals and radiochemicals were as described by Bar-Tana *et al.* (1972). Microsomal palmitoyl-CoA synthetase, prepared and purified as described by Bar-Tana *et al.* (1971, 1972) and having a specific activity of 150 nmol of palmitoyl-CoA formed/min per mg of protein, was used throughout this study.

Methods

Analytical ultracentrifugation. Sedimentation coefficients for the native protein were measured in a Beckman model E analytical ultracentrifuge by using schlieren optics or photoelectric scanning at 280 nm. The native protein was studied in Tris-HCl buffer, pH 7.9, 1 mM-EDTA, 1 mM-dithiothreitol, 0.2 M-KCl and 20% (v/v) glycerol (η_{rel} 1.87; ρ 1.087) at 23°C. Extinction was found to be linearly proportional to protein concentration. Sedimentation coefficients were calculated from the movement of the maximum ordinate of the schlieren curve or the movement of the photoelectric scan's inflexion point. A partial specific volume of 0.7197 mg/ml was calculated from the amino acid composition (McMeekin & Marshall, 1952). The diffusion coefficient of the native protein was determined by schlieren optics (Gosting, 1956) by using the height/area method, with the same buffer and temperature conditions described above.

Sedimentation equilibrium was carried out as described by Yphantis (1964) with the photoelectric scanner at 280 nm. The base-line position on the chart

was corrected by sedimentation depletion of the sample at the end of the sedimentation-equilibrium run (Chervenka, 1969). The native protein was studied in the same buffer as described above. Sedimentation equilibrium of the denatured protein was studied in the presence of 6.4M-guanidinium chloride and 0.1M-2-mercaptoethanol (ρ 1.155) by using the photoelectric scanner at 294 nm. The protein sample was dialysed overnight against 500 vol. of the same medium. The partial specific volume of the protein in the guanidinium chloride medium was assumed to decrease to 0.71 mg/ml (Casassa & Eisenberg, 1964).

Agarose-gel filtration. The molecular weight of the native enzyme protein was determined by gel filtration on a column (60 cm $\times$ 2 cm) of Sepharose-6-B (Andrews, 1970). The elution buffer contained 0.05M-Tris-HCl buffer, pH 7.9, 1 mM-EDTA, 1 mM-dithiothreitol and 20% (v/v) of glycerol. Fractions of 1 ml were collected.

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was carried out as described by Clarke (1964) with Tris-glycine buffer, pH 8.4, and gels of 7.5% (w/v) polyacrylamide containing 0.3% (w/w) of cross-linker. Gels were stained as described by Chrambach *et al.* (1967).

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. The proteins for electrophoresis were incubated at 40°C for 60–120 min at a concentration of about 2 mg/ml, in the presence of 0.02M-sodium phosphate buffer, pH 7.2, 1% (v/v) 2-mercaptoethanol and 1% (w/v) sodium dodecyl sulphate. Before electrophoresis of the protein samples, the column gels were subjected to electrophoresis for 120 min at 5 mA/gel in the presence of 0.1M-sodium phosphate buffer, pH 7.2, 0.1% (w/v) sodium dodecyl sulphate and 0.1% (v/v) 2-mercaptoethanol. Electrophoresis was performed as described by Dunker & Rueckert (1969) in the presence of suitable protein markers, on 5% polyacrylamide-gel columns. Staining and measurement of relative mobilities were performed as described by Dunker & Rueckert (1969).

Amino acid analysis. Protein samples for amino acid analysis were dialysed extensively against doubly distilled water, freeze-dried, dried *in vacuo* at 43°C to constant weight and their nitrogen contents determined by Nesslerization. Hydrolysis was performed in de-aerated 6M-HCl at 110°C for 22 and 69 h (Blackburn, 1968). Amino acid analysis was carried out by means of a Beckman amino acid analyser (model 120B). The recovery of serine and threonine was calculated at zero time of hydrolysis by linear extrapolation. Tryptophan was determined by the method of Beaven & Holiday (1952) with a Cary 14 spectrophotometer. Half-cystine thiol groups were determined as described by Butterworth *et al.* (1967). The protein was reduced in 0.05M-Tris-HCl buffer, pH 7.5, in the presence of 8M-urea and a 10-fold excess

of dithiothreitol. Incubation was carried out overnight under N₂ followed by reaction with excess of 5,5'-dithiobis-(2-nitrobenzoic acid) for 1 h at room temperature. Excess of reagent and reducing agent was removed by gel filtration on a Sephadex G-50 (fine grade) column, and the protein fraction was assayed for thiol groups and nitrogen content and analysed for amino acids. The thiol groups were determined by adding solid dithiothreitol in the presence of 8M-urea and measuring the extinction at 412 nm. The concentration of released reagent was calculated from the molar extinction coefficient of 13 600 (Ellman, 1958).

Electrofocusing studies. Electrofocusing of the enzyme protein was carried out with the LKB apparatus and ampholites as described by Vesterberg (1971).

Lipid analysis. An enzyme fraction of known nitrogen content was dialysed extensively against doubly distilled water, freeze-dried and extracted with chloroform-methanol (2:1, v/v) (Folch *et al.*, 1957). The lower organic phase was analysed for phosphorus content as described by Bartlett (1959) and was subjected to t.l.c. for subsequent determination of phospholipid and neutral lipid. T.l.c. of neutral lipid was carried out in a solvent mixture of light petroleum (b.p. 60–70°C)-diethyl ether-acetic acid (35:15:1, by vol.) (Skipski & Barclay, 1969). T.l.c. for phospholipid was carried out in chloroform-methanol-acetic acid-water (25:15:4:2, by vol.) (Skipski *et al.*, 1964). Spots were detected by means of I₂ adsorption, ninhydrin staining, Ditmer's phosphorus staining, and treatment with periodate-Schiff reagent (Skipski & Barclay, 1969). The separated phospholipid spots detected by means of suitable markers, were analysed for phosphorus as described by Bartlett (1959) and the respective phospholipid contents calculated.

Pulse labelling of the enzyme. This was carried out as described previously (Bar-Tana *et al.*, 1973).

Results

Hydrodynamic properties

The concentration-dependent sedimentation-coefficient curve for the native protein is shown in Fig. 1, giving an $s_{23,w}$ value of 17.8S at infinite protein dilution. However, it should be pointed out that the sedimentation pattern observed was characterized by a broadening of the peak during the run, indicating paucidispersity of the native protein sample. The diffusion coefficient, $D_{23,w}$, determined as described in the Experimental section was found to be $3.55 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ at a protein concentration of $2.0 E_{280}$ units (12 mm light-path). The molecular weight calculated from the sedimentation-diffusion coefficients was 378 000 and presumably reflects a weight-average value. The various molecular species

Fig. 1. Concentration-dependent sedimentation velocity of the native enzyme protein

The experimental conditions were as described in the Experimental section and Kel-F double-sector synthetic boundary cells of 12mm were used. E_{280} was recorded by using the photoelectric scanner. Sedimentation was followed by photoelectric scanning (280nm) of the sample in the 0.12–0.7 E -unit range, and by schlieren optics for the 2.0 E -unit sample. Relative concentrations were corrected for radial dilution during centrifugation.

that constitute the native preparation were analysed by means of conventional sedimentation equilibrium (Yphantis, 1964). As shown in Fig. 2, the species of lowest molecular weight observed was of 170000 under the buffer conditions specified. However, most of the protein seems to undergo association into multiples of the lowest species observed, to yield species of molecular weight 340000, 520000 and 1000000, and possibly even higher.

The average molecular weight of 378000 determined by means of sedimentation-diffusion studies was confirmed by Sepharose 6-B gel filtration of the native protein together with suitable protein markers (Fig. 3). Again, the acyl-CoA synthetase activity, occurred from 170000 daltons upwards. Constant specific activity was observed throughout the acyl-CoA synthetase peak. The apparent molecular-weight range of the acyl-CoA synthetase was several-fold greater than that of apoferritin of approximately the same molecular weight; the association observed in the course of sedimentation-equilibrium studies may also explain the peak broadening observed during gel filtration.

In the presence of 6M-guanidinium chloride and 0.1M-2-mercaptoethanol the protein undergoes dissociation into subunits. The $s_{23,w}$ value of 17.8S observed in the native state decreased under these conditions to 8.0S. Similarly, no peak broadening was observed during sedimentation-velocity experiments. However, sedimentation-equilibrium determination under denaturing conditions again indicated non-homogeneity of the denatured protein (Fig. 4). The

Fig. 2. Sedimentation equilibrium of the native enzyme protein

The experimental conditions were as described in the Experimental section. Overspeeding to 26000 rev./min was followed by performing the run at 10000 rev./min in a double-sector Kel-F cell of 12mm. E_{280} was recorded by photoelectric scanning. Base-line correction was carried out as described in the Experimental section. The molecular weights calculated from the slopes (I–IV) were 165900, 339800, 521000 and 1060000 respectively.

smallest species observed had a molecular weight of 27000 and the largest species one of 167000. It therefore appears that guanidinium chloride causes complete dissociation of the various associated protein species into the smallest species observed under native conditions (166000 daltons), but does not cause complete dissociation to unfolded polypeptide chains. The sedimentation-equilibrium pattern observed appears to indicate the existence of additional molecular species in the 27000–170000-molecular-weight range, but resolution was not good enough to differentiate between these subunits.

Further resolution of the low-molecular-weight species obtained on denaturation of the protein was achieved by means of sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. As shown in Fig. 5, four species, having molecular weights of 168000, 80000, 54000 and 27000, were located on the gel under the conditions described. The relative amounts of the various species were dependent on the 2-mercaptoethanol concentration present during pre-incubation of the protein samples. Preincubation in the presence of 10% (by vol.) 2-mercaptoethanol eliminated the 170000-molecular-weight species, whereas the other molecular-weight species were relatively enriched.

Polyacrylamide-gel electrophoresis in the absence of sodium dodecyl sulphate failed to fractionate the native protein. Activity was found only with the protein band remaining at the start of the column and

Fig. 3. Molecular-weight determination by gel filtration of the native enzyme protein

The experimental conditions were as described in the Experimental section. —, Molecular-weight line; - - - - -, acyl-CoA synthetase activity measured as described by Bar-Tana *et al.* (1971); ·····, apoferritin elution curve. Fractions of 1 ml were collected.

Fig. 4. Sedimentation equilibrium of the denatured enzyme protein

The experimental conditions were as described in the Experimental section. Overspeeding to 38000 rev./min was followed by performing the run at 16000 rev./min, at 25°C, in a charcoal-filled epoxy resin double-sector cell of 12 mm. E_{294} was recorded by photoelectric scanning. Base-line correction was carried out as described in the Experimental section. The molecular weights calculated from slopes (I and II) were 26900 and 167250 respectively.

Fig. 5. Molecular-weight determination by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis

The experimental conditions were as described in the Experimental section. Mobility was measured relative to ovalbumin. The molecular weights for the acyl-CoA synthetase subunit species (I-IV) are 168000, 80000, 54000 and 27000 respectively.

no other protein bands could be located throughout the gel. The native protein could not be introduced into the column electrophoretically by lowering either the polyacrylamide-gel concentration to 2% or the cross-linker concentration to 2% of the polyacrylamide concentration. This behaviour may be explained in the light of the extensive association observed under native conditions.

The native enzyme appeared to be homogeneous on electrofocusing in the pH ranges 1-9 and 4-7. Constant specific activity was observed throughout the electrofocused protein fractions. The pI was 5.5.

The molecular weight of the active monomer unit was determined by labelling the enzyme with radio-

active palmitate under conditions of saturation with both ATP and fatty acid. Pyrophosphatase was introduced during labelling to eliminate reversibility of the first partial step which yields the enzyme-bound intermediate in the absence of CoA. Details of pulse labelling of the fatty acyl-CoA synthetase have been described previously (Bar-Tana *et al.*, 1973). The concentration of the enzyme protein used for labelling was estimated by determination of its nitrogen content (163 μ g of N/mg of protein). As shown in Table 1, 0.405 nmol of labelled intermediate was bound per 68 μ g of protein. Assuming one active site per active monomer it appears that the active monomer unit has molecular weight 168000.

Table 1. *Molecular-weight determination of the active monomer unit*

The reaction mixture for pulse labelling contained 150mM-Tris-HCl buffer, pH 7.8, 4mM-EDTA, 50mM-MgCl₂, 13mM-GSH, 50mM-ATP (where stated), 0.1mM-[9,10-³H]palmitate (500μCi/nmol), 0.2mg of Triton X-100, 1 unit of inorganic pyrophosphatase and 68μg of enzyme in a final volume of 0.11 ml. After a 1 min pulse excess of unlabelled palmitate, ATP (where stated) and CoA were added to final concentrations of 4.35, 50 and 0.4mM respectively. The 'chase' mixtures (0.46ml) were allowed to react for 30s and the radioactive palmitoyl-CoA was isolated and determined. Other experimental details were as described by Bar-Tana *et al.* (1973).

'Pulse' conditions	'Chase' conditions	Amount of [³ H]palmitoyl-CoA formed (nmol/min per 68μg of protein)	Change in amount of [³ H]palmitoyl-CoA formed from labelled intermediate (nmol/min per 68μg of protein)
Enzyme+[³ H]palmitate+ATP	Palmitate+CoA	0.892	0.405
Enzyme+[³ H]palmitate	Palmitate+CoA+ATP	0.487	

Table 2. *Amino acid composition of the acyl-CoA synthetase protein*

Amino acid analysis was carried out as described in the Experimental section.

Amino acid residue	No. of residues per 168000 daltons
Aspartic acid	112.3
Threonine	66.9
Serine	90.7
Glutamic acid	142.1
Proline	68.0
Glycine	96.8
Alanine	85.4
Valine	87.6
Methionine	36.0
Isoleucine	65.9
Leucine	124.7
Tyrosine	40.2
Phenylalanine	58.7
Histidine	29.8
Lysine	91.6
Arginine	60.7
Tryptophan	23.3
Cystine (half)	6.2

Chemical composition

The amino acid composition of the enzyme calculated on the basis of a molecular weight of 168000 is shown in Table 2. The relative abundance of dicarboxylic amino acids is noteworthy and may, together with the phospholipid content, account for the pI of 5.5 determined by means of electrofocusing.

The appearance of glycerophosphorylethanolamine and phosphorylethanolamine in the course of the amino acid analysis indicated that lipid may be bound to the protein moiety of the purified enzyme. Lipid analysis revealed the presence of 49nmol of phospholipid P/mg of protein. Assuming the 170000-molecular-weight species to be the active subunit, it appears that about 8.2mol of phospholipid are

bound/mol of enzyme. T.l.c. analysis of this phospholipid followed by spot analysis gave the following phospholipid composition: phosphatidylinositol, 52%; glycerophosphorylcholine, 26.2%; phosphatidylethanolamine, 21.5% (% of total phospholipid). No other phospholipid nor 'neutral' lipid was detected. Also, free fatty acids were found to be a major lipid component bound to the enzyme protein, but the composition of this fraction has not been studied. The lipid composition described was obtained with several different enzyme preparations.

Discussion

The active monomer of rat liver microsomal long-chain fatty acyl-CoA synthetase appears to have a molecular weight of 170000 and to have a specific lipid content. Whether bound phospholipid is essential for enzyme activity (Pande & Mead, 1968) has yet to be determined. However, it seems worth noting that (in contrast to microsomal membranes) phosphatidylinositol appears to be the major phospholipid component of the purified enzyme. The presence of enzyme-bound phospholipid, despite a butanol-acetone-ether extraction step (carried out before solubilization of the microsomal membrane by deoxycholate), agrees with previously published results (Roelofsen *et al.*, 1971). Especially noteworthy is the presence of enzyme-bound free fatty acid, confirming our previous observation that the purified enzyme fraction is capable of tightly binding palmitate in a non-specific manner (Bar-Tana *et al.*, 1973). This high affinity for fatty acids shown by the enzyme may possibly identify the synthetase with a fatty acid-binding protein suggested previously as a site for the intracellular binding of fatty acids (Reshef & Shapiro, 1965).

The presence of endogenous fatty acids in the purified-enzyme fraction also explains the observation reported previously that the [³²P₂]PP_i-ATP exchange reaction catalysed by the purified enzyme, and inhibited by CoA, is not dependent on the presence of

added fatty acid (Bar-Tana *et al.*, 1972). Similarly, the slow CoA-dependent ATP breakdown into PP_i and AMP, as well as the ATP-dependent CoA disappearance, catalysed by the enzyme in the absence of exogenous fatty acid (Bar-Tana *et al.*, 1973) may rely on small amounts of endogenous fatty acid substrate.

The elucidation of the structure of the 170000-molecular weight species is complicated by the inadequacy of conventional denaturing agents to dissociate membrane proteins completely into their polypeptide chains. In contrast to the dissociation of water-soluble proteins into polypeptide chains produced by either guanidinium chloride or sodium dodecyl sulphate (Tanford, 1968) it appears that membrane proteins are far more resistant (Maddy & Kelly, 1971; Katzman, 1972). To ascertain the subunit composition of the active acyl-CoA synthetase it may be necessary to analyse the amino acid compositions of the various subunits obtained and compare them with the amino acid composition of the native parent unit. It should then be possible to conclude whether the active monomer is composed of six identical polypeptides or whether it consists of a combination of non-identical chains. In this connexion it may be noted that Katzman (1972) has pointed out that electrophoresis of membrane proteins in the presence of sodium dodecyl sulphate may suggest erroneous molecular weights. It is noteworthy that the smallest native species observed has the same apparent molecular weight as the largest species obtained under denaturing conditions.

Association of the native enzyme may explain the purification difficulties (apart from solubilization) that are often encountered during the isolation of membrane-bound enzymes. The more an enzyme associates the lower are the prospects of obtaining enzyme fractions of increasing specific activities during purification by the usual standard procedures. This is obvious in the case of gel filtration, but since association probably also affects net surface charge and charge density as well as the hydrophobic properties of the protein problems may be expected to arise also with ion-exchange or adsorption techniques. For these reasons the presence of detergent (and in some cases also a thiol) throughout the purification procedure may be of critical value in the isolation of membrane-bound enzymes.

The constant specific activity of purified palmitoyl-CoA synthetase observed throughout the fractions obtained by gel filtration, despite the extensive association of the protein units into various molecular-weight species, shows that the specific activity is not affected by the aggregation of the protein. Thus enzyme-purification techniques based on substrate (or substrate analogue) affinity may present an ideal solution to the purification problems encountered when using other procedures.

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