Molecular Weight and Subunit Size of Fatty Acid Synthetase from Rabbit Mammary Gland

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Fatty acid synthetase purified from the mammary gland of the rabbit has a mol.wt. of 968 000 as determined by gel filtration. The enzyme gave one band, corresponding to a mol.wt. of approx. 350 000, on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and phenylmethanesulphonyl fluoride.

Fatty acid synthetase has been isolated from bacteria (Lennarz *et al.*, 1962; Goldman *et al.*, 1963) and can be separated into catalytically active component enzymes and a protein containing 4'-phosphopantetheine by classical protein-fractionation techniques (Vagelos *et al.*, 1966; Prescott & Vagelos, 1972). However, fatty acid synthetases isolated from yeast (Lynen, 1961) and animals (Martin *et al.*, 1961; Hsu *et al.*, 1965; Smith & Abraham, 1970; Maitra & Kumar, 1974; Buckner & Kolattakudy, 1976) do not appear to be readily dissociable in this manner. The fatty acid synthetase isolated from yeast has been reported to consist of a multiple of two polypeptide subunits of similar sizes (Schweizer *et al.*, 1973; Kresze *et al.*, 1976), although earlier reports claimed that the yeast enzyme was dissociable into seven components (Lynen, 1967). The enzyme has been isolated from most animal tissues as a protein of approx. mol.wt. 500 000 which dissociates into subunits of mol.wt. 200 000–250 000 (Stoops *et al.*, 1975; Lornitzo *et al.*, 1975; Buckner & Kolattakudy, 1976), although a report by Bratcher & Hsu (1976) implies that such results may be artifacts and that fatty acid synthetase from chicken liver is a multi-enzyme complex that dissociates into several components on treatment with sodium dodecyl sulphate and urea. However, proteolytic activity promoted by the presence of sodium dodecyl sulphate has been reported to be associated with several purified preparations of fatty acid synthetase from yeast and animal tissues (Schweizer *et al.*, 1973; Stoops *et al.*, 1975; Buckner & Kolattakudy, 1976).

The fatty acid synthetase obtained from the mammary gland of the rabbit synthesizes a large proportion of C_{8:0} and C_{10:0} fatty acids, in contrast with many other animal tissues, and has a mol.wt. of 910 000 as determined by ultracentrifugation (Carey & Dils, 1970). The present paper reports the determination of the mol.wt. of fatty acid synthetase from

rabbit mammary gland by gel filtration, and the determination of the subunit size of the enzyme.

Materials and Methods

Materials

New Zealand White rabbits (albinos; days 15–17 of lactation) were obtained from the Joint Animal Breeding Unit, Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. Sepharose 4B and Sepharose 6B were purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. DEAE-cellulose (Whatman grade DE-52) was obtained from W. and R. Balston (Modified Cellulose) Ltd., Maidstone, Kent, U.K. Calcium phosphate gel and agarose were purchased from BDH Chemicals, Poole, Dorset, U.K. Thyroglobulin, urease (EC 3.5.1.5), bovine serum albumin, pyruvate kinase (EC 2.7.1.40), catalase (EC 1.11.1.6), phosphofructokinase (EC 2.7.1.11), phosphorylase *b* kinase (EC 2.7.1.38), NADPH, malonyl-CoA and phenylmethanesulphonyl fluoride were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Aldolase (EC 4.1.2.7) and ferritin were purchased from Boehringer Mannheim G.m.b.H., West Germany. Globin was purchased from K & K Laboratories, Plainview, NY, U.S.A. All other chemicals were of A.R. grade.

Purification and assay of fatty acid synthetase

Fatty acid synthetase was purified from the mammary glands of lactating rabbits as described by Carey & Dils (1970). Subsequently the enzyme was further purified by chromatography on a column (1.6 cm × 75 cm) of Sepharose 4B equilibrated with 0.5 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 0.5 mM-dithiothreitol. Protein was eluted with the same buffer. The activity of the enzyme was measured as described by Speake *et al.* (1975). Specific activities in the range 1000–1200 nmol of NADPH oxidized/min per mg of

protein were obtained as a routine in the final preparations.

Immunochemical procedures

Antibodies against purified fatty acid synthetase were raised in sheep and fractionated as described by Speake *et al.* (1975). Double-diffusion analyses were carried out by the methods of Ouchterlony (1949) and Piazzi (1969) in 1% (w/v) agarose gels prepared in 0.5M-potassium phosphate buffer, pH 7.0. Rocket immunoelectrophoresis and crossed immunoelectrophoresis were carried out as described by Weeke (1973).

Molecular-weight determination

The molecular weight of the purified fatty acid synthetase was determined by chromatography on a column (0.7 cm × 90 cm) of Sepharose 6B previously equilibrated in 0.5M-potassium phosphate buffer, pH 7.0, containing 1.0 mM-EDTA and 0.5 mM-dithiothreitol. The column was calibrated with several purified proteins. A standard curve of log(molecular weight) against elution volume was constructed. Fatty acid synthetase activity was measured in the eluent from the column after application of 0.3 ml of purified enzyme (2.1 mg) in 0.5M-potassium phosphate buffer, pH 7.0. The activity profile of the enzyme was coincident with E_{280} . Fatty acid synthetase activity was also measured in the eluent from the column after application of 0.3 ml of freshly prepared particle-free supernatants from rat liver, and mammary gland from lactating rat and rabbit. Particle-free supernatants were prepared from homogenates (1:2, v/v) in 0.25M-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 0.5 mM-dithiothreitol.

Phenylmethanesulphonyl fluoride treatment

Before estimation of the subunit molecular weight of fatty acid synthetase the enzyme was treated with phenylmethanesulphonyl fluoride to inactivate serine-proteolytic activity in the preparation (Fahrney & Gold, 1963). A solution of fatty acid synthetase (1 ml) in 0.5M-sodium phosphate buffer, pH 7.0, 1 mM-EDTA and 0.5 mM-dithiothreitol was incubated with phenylmethanesulphonyl fluoride (in 0.1 ml of ethanol) at 20°C for 2–5 h. The molar ratio of enzyme to inhibitor was 1:50000. The solution was dialysed overnight against 0.5M-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 0.5 mM-dithiothreitol. Approx. 95% of fatty acid synthetase activity was inhibited by this treatment.

Subunit-molecular-weight estimation

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out as described by Weber & Osborn (1969) with acrylamide concentrations of 7.5%, 5% and 3.5% (w/v). The 3.5% acrylamide gels were strengthened by the

incorporation of 0.5% (w/v) agarose. The gels containing agarose were prepared at 50°C, allowed to cool, and then irradiated with u.v. light. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and urea was carried out as described by Schweizer *et al.* (1973). Samples of phenylmethanesulphonyl fluoride-treated fatty acid synthetase or untreated fatty acid synthetase were boiled for 2–3 min with sodium dodecyl sulphate (final concn. 2%, w/v) and mercaptoethanol (final concn. 2%, v/v). In some experiments phenylmethanesulphonyl fluoride was added to untreated fatty acid synthetase just before boiling. The samples were allowed to cool, mixed with glycerol and Bromophenol Blue and applied to the polyacrylamide gels. Between 50 and 100 µg of protein was applied to each gel.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate at 3.5, 5 and 7.5% (w/v) acrylamide concentrations was also carried out with several proteins of known subunit size (Darnall & Klotz, 1975). The proteins used were thyroglobulin, urease, globin, pyruvate kinase, bovine serum albumin and alcohol dehydrogenase. The R_x value relative to the tracking dye of the major staining band for each protein was plotted against log(molecular weight) to give a calibration curve for each gel system.

Results

The purity of the fatty acid synthetase preparation was verified by immunochemical methods (results not shown). Immunodiffusion analyses indicated the presence of a single antigen-antibody system, which suggests that no contaminating proteins were present in the preparation of fatty acid synthetase used to raise the antiserum. Crossed immunoelectrophoresis indicated the presence of only one antigen-antibody system, although features which have been interpreted as characteristic of proteolytic modification of antigen (Bjerrum & Bøg-Hansen 1975) were observed.

The mol.wt. of freshly purified fatty acid synthetase was estimated to be $968\,000 \pm 22\,300$ (mean of five determinations ± 2 s.e.m.; Fig. 1). Fatty acid synthetase activity in the particle-free supernatant prepared from mammary gland of a lactating rabbit was eluted with a volume corresponding to a mol.wt. of 980000. Fatty acid synthetase activity in particle-free supernatants obtained from rat liver and rat mammary gland was eluted in volumes corresponding to mol.wts. 430000 and 450000 respectively.

Polyacrylamide-gel electrophoresis of fatty acid synthetase in the presence of sodium dodecyl sulphate and urea showed several bands on staining with Coomassie Blue by the method of Betts & Mayer (1975). However, when the enzyme was treated with phenylmethanesulphonyl fluoride, only one

Fig. 1. *Chromatography of proteins on Sepharose 6B*

Standard proteins: phosphorylase kinase (○); thyroglobulin (Δ); ferritin (□); phosphofructokinase (●); catalase (▲); aldolase (■); bovine serum albumin, monomeric (∇). (1) Elution volume of fatty acid synthetase activity from particle-free supernatant prepared from rabbit mammary gland (mean of two determinations). (2) Elution volume of purified fatty acid synthetase (mean of five determinations). (3) Elution volume of fatty acid synthetase activity from particle-free supernatant prepared from rat mammary gland (one determination). (4) Elution volume of fatty acid synthetase activity from particle-free supernatant prepared from rat liver (one determination).

band was produced, corresponding to the highest-molecular-weight band of the untreated sample.

Polyacrylamide-gel electrophoresis of fatty acid synthetase in the presence of sodium dodecyl sulphate gave several stained bands. The lower-molecular-weight bands were not consistently seen, and varied in position and intensity from one experiment to another. When the enzyme was treated with phenylmethane-sulphonyl fluoride, only one stained band was seen. In 7.5% (w/v) acrylamide gels this band corresponded to a mol.wt. of $355\,000 \pm 20\,300$ (mean of ten determinations ± 2 s.e.m.). In 5% (w/v) acrylamide gels this band corresponded to a mol.wt. of 340 000 (mean of two determinations), and in 3.5% (w/v)

acrylamide gels in the presence of 0.5% (w/v) agarose a value of 300 000 (mean of two determinations) was obtained.

Discussion

Fatty acid synthetase purified from the mammary gland of the lactating rabbit has been shown to have a mol.wt. of approx. 968 000 as determined by gel filtration. This value is in agreement with that of 910 000 obtained by Carey & Dils (1970) by ultracentrifugation. The mol.wt. of fatty acid synthetase from rat mammary gland determined by the gel-filtration method (450 000) is in agreement with that of 478 000 determined by ultracentrifugation (Smith

& Abraham, 1970). The molecular weight of fatty acid synthetase from rat liver measured by the gel-filtration method (430000) is in agreement with that of 350000–420000 measured at a similar ionic strength by ultracentrifugation (Stoops *et al.*, 1975). The similarity of molecular weights of fatty acid synthetase from rat tissues measured by gel filtration and by ultracentrifugation indicates that gel filtration is a reliable method of molecular-weight determination for this protein.

A single subunit of mol.wt. approx. 350000 was seen on polyacrylamide-gel electrophoresis of fatty acid synthetase in the presence of sodium dodecyl sulphate after treatment of the enzyme with phenylmethanesulphonyl fluoride. When the enzyme was not treated with phenylmethanesulphonyl fluoride several bands of lower molecular weight could also be seen. Since no contaminating proteins could be detected in purified fatty acid synthetase by immunodiffusion analysis or on polyacrylamide-gel electrophoresis in the presence of phenylmethanesulphonyl fluoride, it may be speculated that proteolytic activity is intrinsic in the enzyme itself. This could be due to an 'active serine' present in each polypeptide, such as that demonstrated in fatty acid synthetase from the uropygial gland of the goose (Kolattakudy *et al.*, 1976) which is normally involved in fatty acid chain termination by deacylation. This site is inactivated in the enzyme from pigeon liver and rat liver by phenylmethanesulphonyl fluoride (Kumar, 1973). Alternatively, very small amounts of contaminating proteinase(s) may be present in the purified enzyme preparation. Medium chain deacylase has been immunochemically detected in purified fatty acid synthetase preparations from rabbit mammary gland (L. Chivers, R. Dils & R. J. Mayer, unpublished work).

Crossed immunoelectrophoresis of fatty acid synthetase in low-ionic-strength buffers gave results which can be interpreted as due to proteolytic degradation of the enzyme (Bjerrum & Bøg-Hansen, 1975). The dissociation of fatty acid synthetase in low-ionic-strength buffers (Carey & Dils, 1970; Stoops *et al.*, 1975) may be accompanied by limited proteolysis of the enzyme complex.

The incorporation of urea into the polyacrylamide-gel system did not result in dissociation of the enzyme into several components in the presence of phenylmethanesulphonyl fluoride, unlike the enzyme obtained from chicken liver (Bratcher & Hsu, 1976).

The fatty acid synthetase from the mammary gland of the rabbit is clearly different in size from that in many other animal tissues. The ratio of enzyme molecular weight to subunit molecular weight obtained in the experiments (2.7–3.2:1) suggests a stoichiometry of three polypeptide subunits per molecule of enzyme, an uncommon, though not unique, ratio (Darnall & Klotz, 1975).

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