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CHARACTERIZATION OF SUBSPECIES FROM A FUNGAL FATTY ACID SYNTHETASE

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Summary

Fatty acid synthetase from the filamentous fungus, *Aspergillus fumigatus* dissociates during gel filtration on Sepharose 6B into two differently sized subspecies with mol. wt. approx. $1.5 \cdot 10^6$ and $8 \cdot 10^5$. After elution, they readily reform intact molecules, as determined by their enzymic activity (overall synthetase and 3-oxoreductase activities were measured), sedimentation coefficient and appearance in the electron microscope.

Synthetase was cross-linked with dimethyl suberimidate and the resultant protein did not dissociate on Sepharose 6B. The two smaller species which were eluted after chromatography of untreated enzyme were also fixed by reaction with this reagent. They did not reform intact molecules of synthetase and were characterized by electron microscopy as large and small circular aggregates; the low molecular weight form also contained tetrameric structures which exhibited cyclic symmetry. The composition of the two species derived during dissociation was, therefore, confirmed as eight and four polypeptides, respectively; each contained polypeptides A and B.

It is proposed that the intact fungal synthetase of composition A_6B_6 comprises three equivalent loops of protein, each of which contain four polypeptides, presumably with composition A_2B_2; the molecular weights of A and B are 207 000 and 201 000, respectively. During filtration on Sepharose 6B, two such loops remain associated to form a large circle, leaving the other four polypeptides to rearrange themselves into a small circle or tetramer.

Abbreviation: PMSF, phenylmethylsulphonyl fluoride.

Introduction

Fatty acid synthetase has been isolated from the filamentous fungus, *Aspergillus fumigatus* [1] and purified to high specific activity (3000—5000 munits/mg protein) [2]. The pure enzyme has a sedimentation coefficient of 42 S, in common with that from yeast [3], but in the course of its isolation a number of larger forms have been noted. For example, an active heavy form (60 S) has been resolved by sucrose gradient centrifugation and observed during analytical ultracentrifugation. Even heavier forms of active enzyme (with sedimentation coefficient greater than 100S) have been detected in crude extracts or partially purified preparations [2]. However, these very large components (which may arise after binding of synthetase to membranous material) have been difficult to visualise in electron micrographs but this may have arisen because of problems arising from the staining techniques, such as concentration and desiccation of the samples.

Since the nature of the heavy material could not be readily seen, an attempt was made to confirm the existence of the various synthetase species with different molecular weight by gel filtration after passage through Sepharose 6B. It was considered that the 'heavy' forms would be excluded from the gel and eluted in the void volume, whereas the normal 40 S-form (mol. wt. $2.3 \cdot 10^6$) [3] of the purified enzyme would be included within the beads and slightly retarded. However, as a result of these experiments, detailed information concerning the structure of the fungal synthetase and its dissociated subcomplexes was found. Previous work has shown that the synthetase partially dissociates into two differently sized subunits, after aging; these were detected by electron microscopy [2] but were not separated and no information was available concerning their molecular weight.

Schweizer and colleagues [4—6] and others [2,7] have recently demonstrated that fatty acid synthetase in yeast and filamentous fungi exists in a polymeric form, comprising two large multifunctional polypeptides with overall composition A_6B_6, but the arrangement of these 12 polypeptides within the complex has not been identified. This paper describes the results obtained from gel filtration of the synthetase on Sepharose 6B which has helped to characterize two subspecies in terms of their molecular weight and composition, and appearance in the electron microscope. A model for the structure of this fungal enzyme is presented. Preliminary results describing some of this work have been reported [8].

Materials and Methods

Coenzymes, substrates, dithiothreitol and PMSF were obtained from Sigma London Chemical Co. Ltd., and bovine serum albumin and chemicals of reagent grade and AnalaR standard from B.D.H. Ltd. (Poole, Dorset). DEAE-cellulose and Sepharose 6B were obtained from Whatman Ltd. (London) and Pharmacia (Uppsala, Sweden), respectively. Standard proteins and *S*-acetoacetyl-*N*-acetylcysteamine were supplied by Sigma and Boehringer Mannheim GmbH. (F.R.G.), respectively.

Cultural conditions. The organism used in this work was *A. fumigatus* (I.M.I.

85393) which was obtained from the Commonwealth Mycological Institute (Kew, Surrey). Stock cultures, preparation of conidia, submerged cultures (initially in 50 ml portions and then transferred to flasks of 2 l capacity containing 500 ml of fresh medium) and mycelium were obtained as described previously [1,2,9].

Purification of enzyme. Fatty acid synthetase was purified from freeze-dried mycelium by $(NH_4)_2SO_4$ fractionation, ultracentrifugation, chromatography on DEAE-cellulose followed by ultrafiltration through an Amicon XM-300 membrane [2], and (usually) sucrose gradient centrifugation. This was performed by passage through 30 ml of a linear 10—40% (w/v) sucrose gradient after centrifugation for 22 h at 22 000 rev./min in a Beckman SW 25.1 rotor. The resulting protein was considered pure and virtually free from proteolytic damage, on the basis of its SDS-polyacrylamide gel electrophoresis [10] pattern and its behaviour on subsequent sucrose gradient centrifugation, and was subjected to gel filtration on Sepharose 6B.

The $t_{1/2}$ for the hydrolysis of PMSF in 0.2 M potassium phosphate buffer at 20°C is 50 min (Fig. 1); it proved difficult to dissolve at lower temperatures and eventual solution was balanced by increased hydrolysis. Use of this inhibitor of serine proteases is therefore only effective in freshly made-up buffer if used rapidly. Accordingly, it is essential to use fresh buffer for the initial extraction and also for the resuspension of the pellets derived from $(NH_4)_2SO_4$ treatment and ultracentrifugation, respectively, since hydrolysis of PMSF is almost complete in approx. 3 h. It was considered that proteases were removed by this stage since incubation of purified synthetase under denaturing conditions (treatment with SDS at 25°C for 1 or 3 days) did not result in any proteolysis; only the two high molecular weight polypeptides were observed after SDS-polyacrylamide gel electrophoresis.

Improved use of PMSF and better filamentous growth of mycelium in this work gave rise to synthetase containing polypeptides of higher molecular

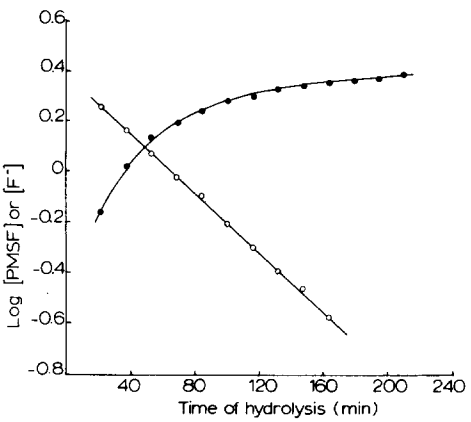

Fig. 1. Hydrolysis of PMSF in phosphate buffer. A solution of PMSF in isopropanol (0.25 M) was slowly injected into 0.2 M phosphate buffer, pH 7.0, containing 1 mM K · EDTA and 1 mM dithiothreitol at 20°C with vigorous stirring, to give a final concentration of 2.5 mM. F^- concentration was determined using a fluoride electrode and the amount of PMSF remaining was calculated by difference. Fluoride (mM), (●———●); PMSF remaining, (○———○).

weight than previously reported [2,5]. The mol. wt. determined were 207 000 ± 6000 and 201 000 ± 5000 (11), respectively, using kidney microvillar proteins [11] and RNA polymerase as standards for SDS-polyacrylamide gel electrophoresis [10].

Enzyme and protein assays. Synthetase activity and protein content were determined as described previously [2]. The 3-oxoreductase component of this fungal enzyme was assayed by the procedure of Lynen [12] except that the concentration of the substrate (*S*-acetoacetyl-*N*-acetylcysteamine) used was 12 mM, the value corresponding to maximum activity; this reductase activity (with the K_m value for this substrate 6.5 mM) is considerably more stable than overall fatty acid synthetase. Protein distribution from sucrose gradients and Sepharose 6B was measured after reaction with fluorescamine [13]. Cross-linked protein was detected by the method of Lowry et al. [14].

Calibration of Sepharose 6B. A slurry of Sepharose 6B was suspended in 0.2 M phosphate buffer, pH 7.0, and used routinely in a column of dimensions 103 × 0.9 cm as described [8]. Marker proteins (1.5 mg/ml) and sample were dissolved in the same buffer (1.5 ml) and clarified before being applied to the column. Aliquots (0.2 ml) from the eluted fractions (1 ml) were assayed in duplicate for protein by reaction with fluorescamine and suitable fractions were assayed for synthetase and reductase activity when appropriate.

The markers were all eluted as symmetrical peaks of protein, and their elution position was consistently maintained throughout these investigations.

Electron microscopy. Fatty acid synthetase (0.1—0.5 mg/ml) and its dissociated species (0.05—0.1 mg/ml) were examined in a Philips EM 300 instrument after contrasting with a 2% (w/v) aqueous solution of uranyl acetate.

Treatment with cross-linking reagents. Initial attempts to cross-link the synthetase with glutaraldehyde resulted in total precipitation even at low concentrations of protein (1—10 μg/ml). Dimethyl suberimidate, which has been used to cross-link various proteins [15,16] by reaction with free amino groups, proved more successful. Synthetase (3 mg; 0.12 mg/ml) was treated with this reagent (5 ml; 0.5 mg/ml) in 0.2 M phosphate pH 8.0, prepared immediately before use, at room temperature with constant stirring, to give a final concentration of 0.08 mg/ml [17,18]. The reaction was maintained for 3 h when phosphoric acid (0.1 ml) was added to remove excess reagent. Precipitated material was removed by low-speed centrifugation and the supernatant (after concentration against poly(ethylene glycol)) applied to a sucrose gradient, as described above, to obtain cross-linked synthetase. A protein (40 S) was obtained (10% overall yield) the identity of which was established after electron microscopy as synthetase with dimensions similar to those of the native enzyme. Fluorescamine assay confirmed that available amino groups had reacted completely.

Fractions obtained from gel filtration were similarly treated immediately after elution. Dimethyl suberimidate (0.25 ml; 4 mg/ml in 0.2 M phosphate, pH 8.0) was added to these fractions, the pH of which had been increased to 8.0 by titration with 10 M KOH, and left at room temperature for 3 h.

Results

Synthetase was partially purified to the first ultracentrifugation step when it exists predominantly as a very heavy form [2]. This was applied to Sepharose 6B and was completely excluded from the column since it appeared in the void volume (Fig. 2). Synthetase purified by the usual procedures [2] (with a sedimentation coefficient of 40 S) was retarded slightly and began to appear as a wide band some fractions later (Fig. 2). However, a further smaller peak of protein and enzyme activity was also eluted in this preparation which corresponded to material with considerably lower molecular weight. Electron micrographs of the material from the first peak showed intact molecules of synthetase, but the second peak contained additionally large and small circular species, characteristic of the structures apparent in aged preparations [2]. Analytical ultracentrifugation of the original sample showed that only 40 S-enzyme was present with no indication of material with lower S values.

Dissociation of aged and fresh synthetase on Sepharose 6B

Enzyme was aged for 3 weeks to promote dissociation [2] and subjected to gel filtration under standardized conditions (see Materials and Methods and Ref. 8) to obtain material for examination. It gave rise to two major regions of protein which were eluted close to the positions found for phosphorylase kinase and thyroglobulin. Accordingly, their molecular weight was considerably less than $2 \cdot 10^6$ and $1 \cdot 10^6$, respectively.

Freshly prepared synthetase which contained only intact molecules (as verified by electron microscopy) was tested by this procedure for comparison. Surprisingly, however, this gave rise to the same elution pattern which exhibited almost total dissociation [8]. Under the same conditions, phos-

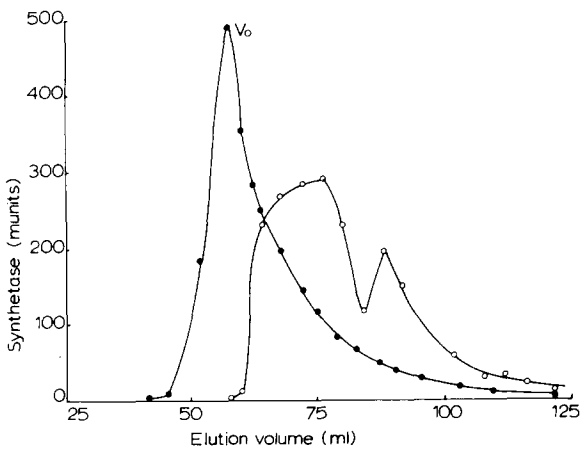

Fig. 2. Chromatography of fatty acid synthetase on Sepharose 6B. Synthetase was purified as described in the text and applied to a column (60 × 2.5 cm) of Sepharose 6B at 4°C, equilibrated with 0.2 M potassium phosphate, pH 7.0, containing 1 mM K · EDTA and 1 mM dithiothreitol. Fractions (4 ml) were eluted with the same buffer at a flow rate of 12 ml/h. V_0, void volume; ●———●, enzyme purified by one ultracentrifugation step; ○———○, enzyme further purified by chromatography on DEAE-cellulose and ultrafiltration.

phorylase kinase (an oligomer comprising four ($\alpha\beta\gamma\delta$) units [19]) and aldolase did not dissociate and were all eluted as single symmetrical peaks.

The molecular weight of the two proteins eluted corresponded to approx. $1.5 \cdot 10^6$ and $8 \cdot 10^5$ [8]. Electron microscopic examination of these samples both showed essentially whole molecules. The expected circular subunits were not present to any significant extent in either case, except occasionally in some samples.

Freshly prepared and aged synthetase (3 weeks at 4°C) obtained from sucrose gradient centrifugation was also tested for dissociation by application to a further sucrose gradient. Both fresh and aged samples sedimented as symmetrical peaks of protein with an unchanged $s_{20,w}$ of approx. 40 S, the value for intact molecules of yeast [3] or fungal enzyme [2]. Smaller species were not detected.

Recovery of enzymic activity

In further experiments performed with greater quantity of protein which had been rigorously purified (additional passage through a sucrose gradient), enzymic activity of the eluted fractions was determined by the 3-oxoreductase assay. The results show that the profiles for reductase activity and protein content followed each other extremely closely throughout elution (Fig. 3). The second peak, however, was considerably less extensive in area (approx. 1 : 3 based on protein and 1 : 6 based on enzymic activity) than the earlier peak which corresponded to the larger protein species. A small inflexion was noted here (and in Ref. 8) in the leading edge of the peak, in a position expected for unchanged synthetase. All the fractions eluted posssessed reductase activity (recovery approx. 75%). Proteins from both peaks were examined by SDS-polyacrylamide gel electrophoresis and contained essentially only polypeptides A and B.

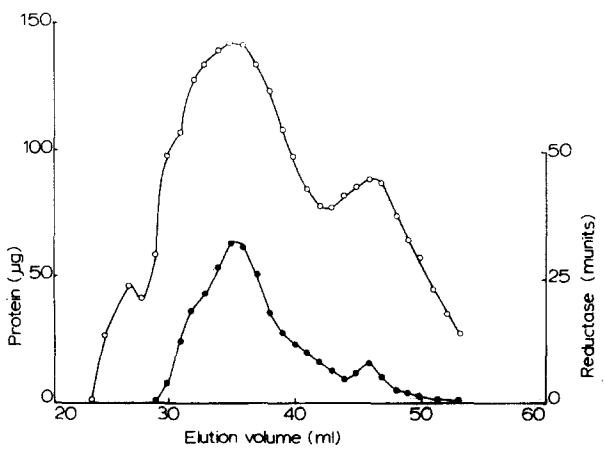

Fig. 3. Chromatography of synthetase on Sepharose 6B and enzymic assay of fractions eluted. Synthetase was purified by procedures including the sucrose gradient step [2]. Appropriate fractions containing synthetase were concentrated by dialysis against 0.2 M potassium phosphate, pH 7.0, containing 1 mM K · EDTA, 1 mM dithiothreitol and 30% poly(ethylene glycol) and applied (1 ml; 3.0 mg) to the column. Fractions (1 ml) were assayed for protein (○———○) by reaction with fluorescamine [13] and reductase activity (●———●).

Sucrose gradient centrifugation of eluted synthetase

Additional experiments were carried out to confirm the nature of the material eluted. Fractions eluted around the region from each of the two peaks all possessed 3-oxoreductase and overall synthetase activity, and were taken and bulked separately. The sedimentation pattern for both groups of concentrated material corresponded to intact molecules of synthetase with $s_{20,w}$ of 40 S (Fig. 4). No smaller proteins were present in the gradients obtained from either set of fractions.

Effect of protein concentration on elution profile

The effect of variation in synthetase concentration on the pattern of dissociation was further examined. An initial higher concentration (4.5 mg/ml; 2 μM) did not effect the pattern obtained and a characteristic elution profile resulted (Fig. 5). A lower protein concentration (approx. 0.2 μM) caused the eluted material to spread more widely but the pattern obtained showing two regions of lower molecular weight protein was again repeated (Fig. 5). A relatively large amount of enzyme aggregated at this low concentration and eluted at the void volume.

Recovery of protein at all concentrations in these experiments and others was approx. 90%.

Cross-linking of synthetase

The results described in Figs. 3 and 5 demonstrate that fatty acid synthetase dissociates extensively during gel filtration. Attempts were therefore made to assess the behaviour of a cross-linked form of enzyme in which the individual polypeptides were covalently linked to each other. This material should conform to an elution pattern expected for a protein with a molecular weight over $2 \cdot 10^6$.

Cross-linking with dimethyl suberimidate (see Materials and Methods) successfully yielded a modified form of synthetase which was applied to Sepharose

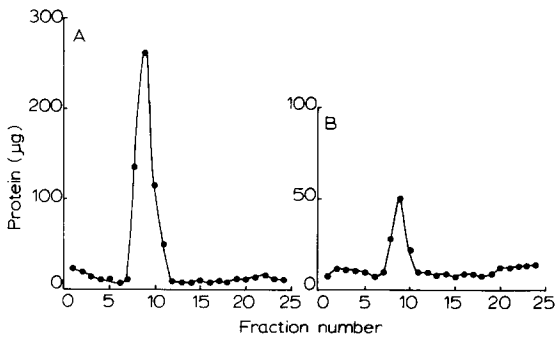

Fig. 4. Sucrose gradient centrifugation of synthetase obtained from Sepharose 6B. Synthetase fractions were obtained from chromatography on Sepharose 6B but material with molecular weight over $1.7 \cdot 10^6$ and four fractions between the two peaks were discarded. Each region around the two peaks was concentrated separately by dialysis against poly(ethylene glycol) (Fig. 3) and subjected to sucrose gradient centrifugation [2]. Fractions (1 ml) were assayed for protein by reaction with fluorescamine [13]. A, represents protein derived from fractions 34—38 eluted from the standard column (see e.g., Fig. 3) and B, fractions 44—50.

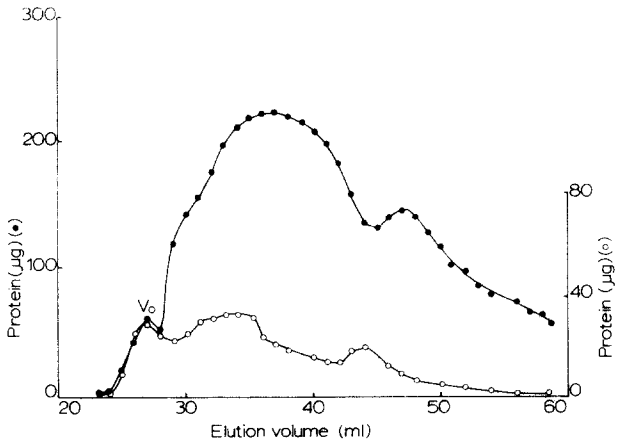

Fig. 5. Effect of protein concentration on chromatography of synthetase on Sepharose 6B. Samples (1 ml) containing 4.5 mg (•———•) or 0.45 mg (○———○) of synthetase were applied separately. Fractions (1 ml) were assayed for protein [13]. V_0 represents the void volume.

6B. This resulted in the elution of a single symmetrical peak of protein, corresponding to a molecular weight of approx. $2.4 \cdot 10^6$. No peaks corresponding to proteins with lower molecular weight were found, in contrast with the profiles obtained with untreated synthetase.

Cross-linking of eluted synthetase

Similar attempts were made to fix the peak fractions from untreated synthetase immediately after elution from Sepharose 6B, to examine the structure of the subcomplexes by electron microscopy. Fractions corresponding to the two peak regions of protein were taken for this purpose.

Electron microscopic examination of the soluble cross-linked material from the first set of treated fractions (mol. wt. approx. $1.5 \cdot 10^6$) revealed mainly large circular species (Fig. 6A) which were similar (but not identical) in appearance to the large circles detected in aged preparations (diameter 18—20 nm) [2]. Few small circles were present in this sample and virtually no reassociation to whole molecules had taken place.

Similar electron microscopic examination of the cross-linked material derived from the fractions containing the lower molecular weight species (approx. $8 \cdot 10^5$) was performed (Fig. 6B). Many of the structures visible were typical of the small circles (diameter 12 nm) [2] but the main species present were distinct tetramers in which the protomeric units were directed towards the corners of a square (cyclic symmetry) [20]. Again, few large circles or whole molecules could be seen in the preparation.

Discussion

The elution profile of fatty acid synthetase from *A. fumigatus* after gel filtration indicates that this enzyme consistently dissociates, over a wide range of concentration, into two differently sized species with mol. wt. approx. $1.5 \cdot$

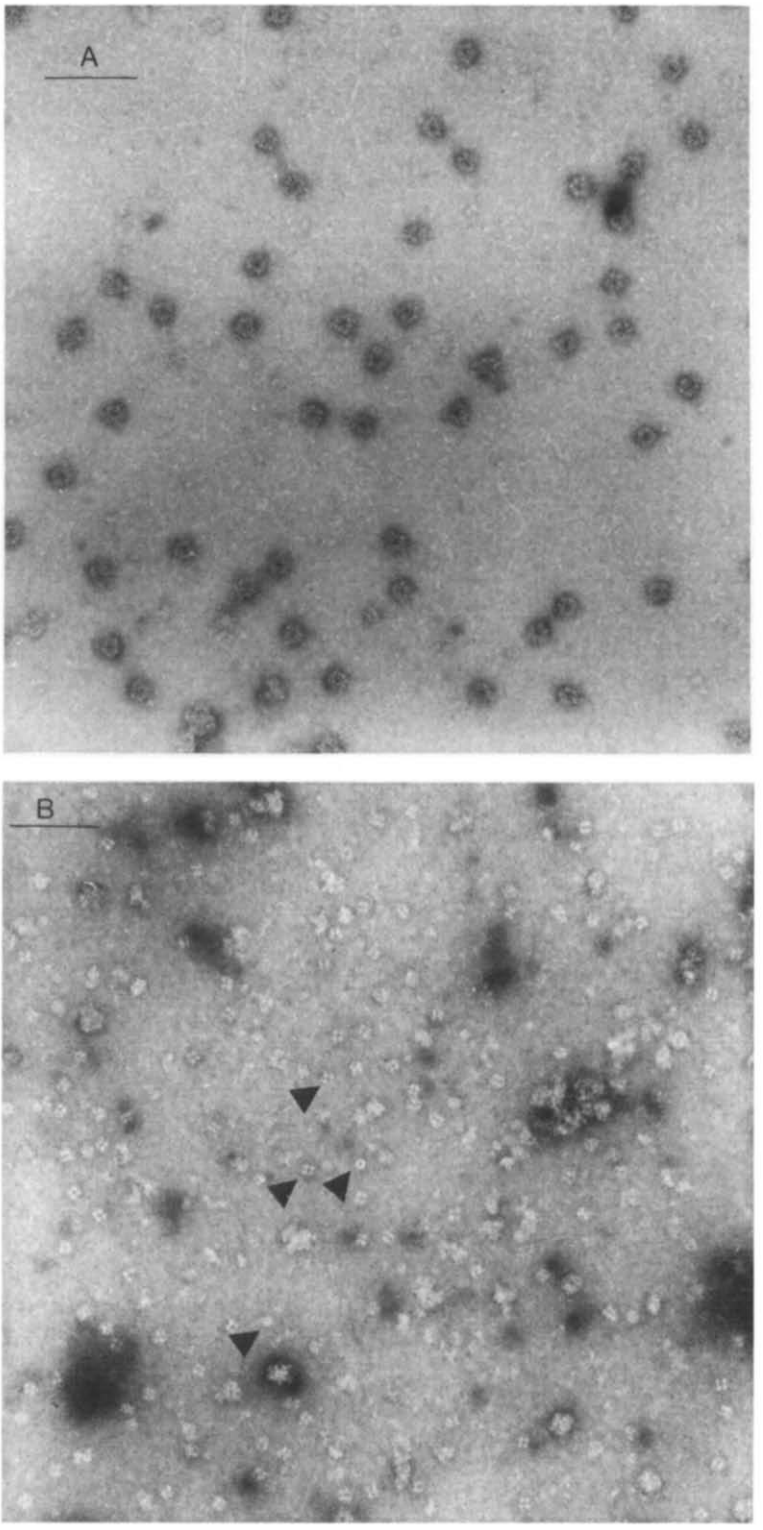

Fig. 6. Electron micrographs of synthetase eluted from Sepharose 6B and cross-linked immediately with dimethyl suberimidate. Fractions corresponding to mol. wt. $1.5 \cdot 10^6$ and $8 \cdot 10^5$ (two in each case) were taken for this purpose (0.8 from 1 ml) and were titrated immediately after elution to pH 8.0, followed by addition of suberimidate solution. The soluble material obtained after this treatment was examined. A, represents protein derived from the first (higher molecular weight) subspecies eluted and B, the second subspecies. Fractions were stained with 2% (w/v) uranyl acetate and the markers point to small circular species and square tetrameric units in B. The calibration line corresponds to 100 nm.

10^6 and $8 \cdot 10^5$. Dissociation is presumably encouraged by the ready entry of the smaller species into the gel beads, which removes them from the 'pool' and causes further dissociation. The first peak contains significantly more enzymic activity and protein than the 'low' molecular weight component, indicating that this larger protein is the favoured species travelling through the gel. In contrast, application of cross-linked synthetase resulted in the elution of a single protein, with a molecular weight corresponding to that of the native enzyme. Moreover, dissociation of the standard proteins used, including phosphorylase kinase ($(\alpha\beta\gamma\delta)_4$), did not occur, but each of these is considerably smaller than the synthetase and readily enters the gel.

The results reported here indicate that both subspecies of the synthetase may readily and independently reassociate under a variety of conditions. Assessment of enzymic activity further confirms the presence of potentially active polypeptides A and B in both subcomplexes. It is suggested, therefore, that this synthetase exists in equilibrium with its dissociated species, with the undissociated form predominating. When either of the dissociated subspecies, which are presumably present in trace amounts, are removed from the system by inclusion in the Sepharose gel, more synthetase dissociates to maintain the equilibrium. Conversely, the dissociated species may readily reassociate to reform synthetase molecules. Thus, whenever the fractions are examined by electron microscopy, which involves a concentration process, mainly intact molecules are seen. Similarly, each fraction shows overall synthetase and reductase activity but these assays are performed at 30 °C in the presence of all substrates. It has been established that hydrophobic interactions are responsible for the stability and overall conformation of the native synthetase from pigeon liver [22], rat mammary gland [23], and also *A. fumigatus* [2]. The vertebrate synthetases are cold-labile and dissociate at 0 °C into half molecular weight species with no overall activity.

The values obtained from the molecular weight data correspond well with species containing eight and four polypeptides (mol. wt. approx. 200 000). Moreover, visual observations made directly from electron micrographs confirm that the cross-linked small subcomplex contains four polypeptides. The proteins observed correspond to the large and small circular subunits previously detected in preparations of aged synthetase [2]. This view is summarized in Table I.

Fungal synthetases (including the yeast enzyme [5,21]), therefore, comprise 12 multifunctional polypeptides, with an overall composition A_6B_6. This information, together with that obtained from negative staining [2,3] and shadowing techniques [2], suggest that they are arranged in the native complex in both filamentous fungi and yeast as three equivalent loops of protein, each with probable composition A_2B_2 and assembled in the order ABBA (or BAAB). On partial dissociation, two such loops remain associated to form the large circular subspecies (A_4B_4) (which would be symmetrical), whereas the third loop rearranges itself into the more favourable conformation of a small circle, or tetrameric structure with cyclic symmetry.

Although dissociation of the synthetase would theoretically produce equimolar proportions of the two subspecies (A_4B_4 and A_2B_2), with protein content in the ratio of 2 : 1, the larger subspecies predominates during elution.

TABLE I

PROPERTIES OF FATTY ACID SYNTHETASE AND ITS SUBSPECIES

A and B represent the two polypeptide components with mol. wt. 207 000 and 201 000, respectively.

Structure	Appearance in electron micrographs	Composition	10^{-6} × mol. wt.	10^{-6} × observed mol. wt.
Fatty acid synthetase	Intact molecules	A_6B_6	2.45	2.40
Large subspecies	Large circles	A_4B_4	1.63	1.48 ± 0.04 (5)
Small subspecies	Small circles; square tetramers	A_2B_2	0.82	0.78 ± 0.05 (5)

Thus dissociation of the synthetase may be presented as follows:

$$A_6B_6 \rightleftharpoons A_4B_4 + A_2B_2 \overset{?}{\rightarrow} 2\,A + 2\,B$$
$$\downarrow\uparrow$$
$$2\,A_2B_2$$

Further dissociation into individual polypeptides may occur but no evidence is available to support this, since little protein is recovered at the expected elution volumes.

Other examples have been reported in which proteins exist as rapidly reversible monomer-polymer equilibria, such as nerve growth factor, for which a monomer-dimer equilibrium is found, with the monomeric species predominant at very low concentration [24]. Further, the dissociation equilibrium of malate dehydrogenase from porcine mitochondria moves in favour of dimer formation at low enzyme concentration in the presence of coenzymes [25].

Gel filtration of fatty acid synthetase from *Corynebacterium diphtheriae* on Sepharose 4B suggests a molecular weight of around $2.5–3 \cdot 10^6$, with no indication of dissociation [26]. (The enzyme from the related *Mycobacterium smegmatis* [27] and *Brevibacterium ammoniagenes* [28], however, has a considerably lower mol. wt. (approx. $1.4 \cdot 10^6$).) Despite the apparent similarity in molecular weight to the fungal enzyme, the different behaviour of the synthetase may be explained by proposing that the molecules more readily enter the larger beads present in the Sepharose 4B. Moreover, the enzyme from *B. ammoniagenes*, which has a lower mol. wt. ($1.2 \cdot 10^6$), migrates through Sepharose 6B as a single protein species [28], as does phosphorylase kinase in this study.

Further evidence in support of the composition of the subspecies has recently been gained with the yeast synthetase [21]. This enzyme may exist as A_6B_6 and A_2B_2 species at high protein concentration but is found predominantly as A_2B_2 at low protein concentration. In the presence of all substrates, the A_6B_6 species was the active form and smaller oligomers were either inactive or not produced. A_4B_4 species were not detected [21].

The structure of fatty acid synthetase which permits dissociation into smaller subspecies may be of special significance in filamentous fungi. This phenomenon may be related to their ability to form an aromatic synthetase responsible for the synthesis of acetate-derived phenols [29,30]. This protein has many enzymic functions in common with fatty acid synthetase [30] but has a considerably lower molecular weight in *Penicillium patulum* [31]. It is

possible that this enzyme may be derived from fatty acid synthetase by dissociation.

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