

DISSOCIATION AND RECONSTITUTION OF THE STABLE MULTIENTZYME COMPLEX FATTY ACID SYNTHETASE FROM YEAST

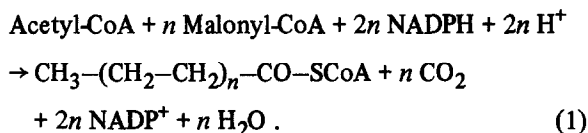
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1. Introduction

In the course of studies on fatty acid synthesis in this laboratory, a stable multienzyme complex from yeast was isolated which produces palmitoyl- and stearoyl-CoA according to eq. (1):



This multienzyme complex behaves during protein fractionation, centrifugation, and electrophoresis as a single protein particle of molecular weight 2.3×10^6 (ref. [1]).

The synthesis of fatty acids requires at least six different enzymatic steps which have been demonstrated with the help of model substrates [2]. After transfer of acetyl- and malonyl-residues from acetyl-CoA and malonyl-CoA to thiol acceptor groups of the enzyme complex, condensation results in acetoacetyl-enzyme. Reduction with NADPH to β -hydroxybutyryl-enzyme, dehydration to crotonyl-enzyme and flavin catalyzed reduction to butyryl-enzyme ends the first cycle of synthesis. The enzyme-bound butyryl residue is further elongated to the C_6 -acid by introduction of another malonyl-unit and a second cycle of reactions. After seven or eight such cycles the synthetic process is terminated by transfer of palmitoyl- or stearoyl-residues to coenzyme A [3].

Abbreviation:

DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent).

In order to obtain some insight into the organisation of the multienzyme complex and the stoichiometry of its various components, attempts have been made over the past several years to split the complex into smaller units. Treatment with urea, guanidine hydrochloride, detergents, acids and bases leads to disruption of the complex, but simultaneously to irreversible loss of enzymatic activity. This paper reports a simple method found recently to promote reversible dissociation of the complex.

2. Materials and methods

The purification and assay of fatty acid synthetase followed procedures described in ref. [4].

5,5'-dithio-bis-(2-nitrobenzoic acid) was obtained from Fluka. Other commercially available preparations used were CoASH, NADPH and serumalbumin from C.F.Boehringer & Söhne, Mannheim, and LiCl and NaCl from Merck, Darmstadt.

Acetyl-CoA, malonyl-CoA and S-acetoacetyl-N-acetylcysteamine were prepared according to refs. [5,6,7], respectively.

Measurement of the β -ketoacid reductase activity was carried out according to ref. [1].

For sedimentation studies a Beckman model E analytical ultracentrifuge fitted with Schlieren optics was available.

Buffer system 1 described by Maurer [8] was used in analytical electrophoresis in 3.4% polyacrylamide gel.

Dissociation of the synthetase occurred after the following treatment. An enzyme solution 0.1 M in phosphate buffer pH 6.5 or 0.1 M in Tris pH 7.5 with

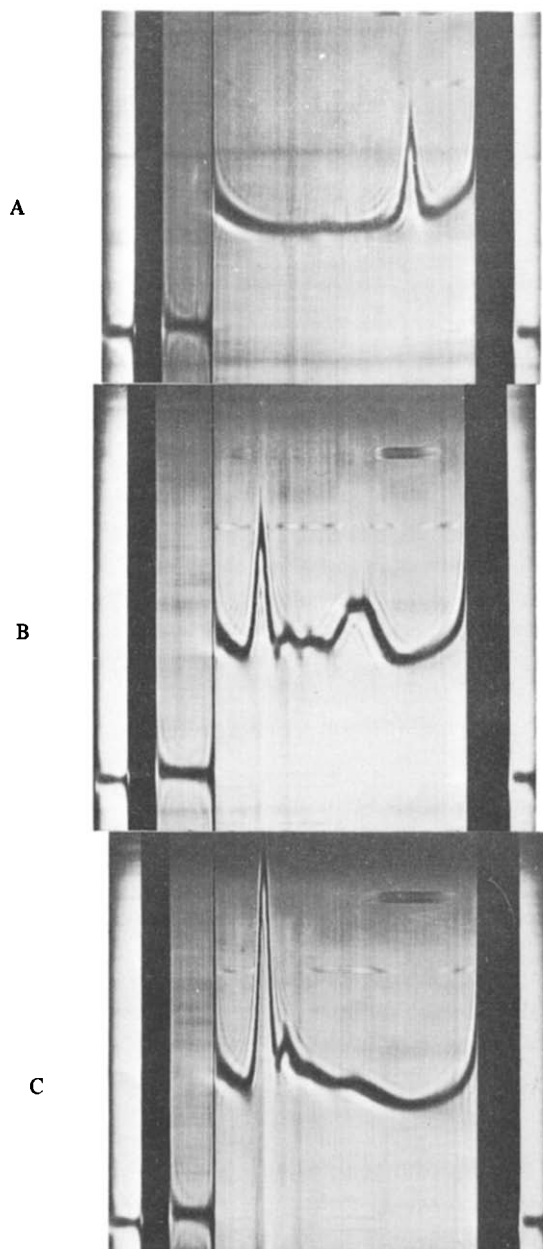


Fig. 1. Changes in sedimentation behaviour after freezing of enzyme solutions 1 M in LiCl.

- (A) Fatty acid synthetase (3 mg/ml) in 1 M LiCl.
 (B) Fatty acid synthetase (10 mg/ml) in 1 M LiCl after a single freezing and thawing.
 (C) Same preparation as in (B) after a second freezing and thawing.

All photographs were taken 14 min after reaching the maximum speed of 59 780 rpm, 20°C. Sedimentation from left to right.

10–15 mg protein per ml was mixed with NaCl or LiCl to a concentration of 1 M and then frozen in a dry-ice/alcohol bath at approximately -70°C . After thawing at room temperature the process was repeated.

An enzyme solution inactivated by the above treatment can be reactivated as follows: the solution is diluted at least five-fold with 0.1 M phosphate buffer pH 7.5 containing 40 mM cysteine and incubated at 20°C . Alternatively, the salt solution can be dialysed against the same buffer, containing in addition 10^{-4}M FMN.

3. Results

Native fatty acid synthetase can be dissolved in 1 M NaCl or LiCl without loss of its enzymatic activity or change in its sedimentation and electrophoretic behaviour. The freezing of such a solution two or more times for fifteen minutes at -70°C and thawing at room temperature results in a drastic alteration of enzymatic and physical properties.

Fig. 1 shows the sedimentation behaviour in an analytical ultracentrifuge of native and treated en-

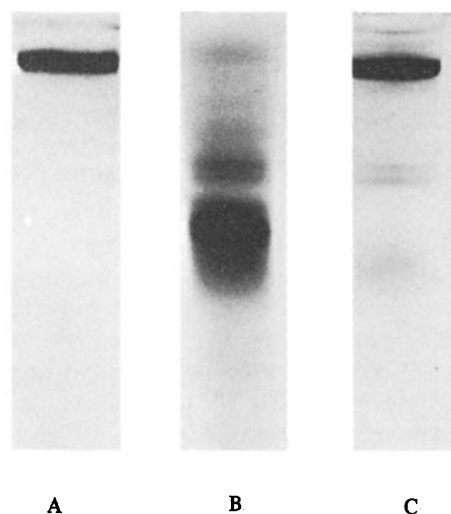


Fig. 2. Electrophoresis in 3.4% polyacrylamide, pH 9.5.

- (A) Fatty acid synthetase in 1 M LiCl.
 (B) Same preparation after two freezings and thawings.
 (C) Same as (B) after reactivation by dilution (see experimental section).

100 μg protein was applied in each case.

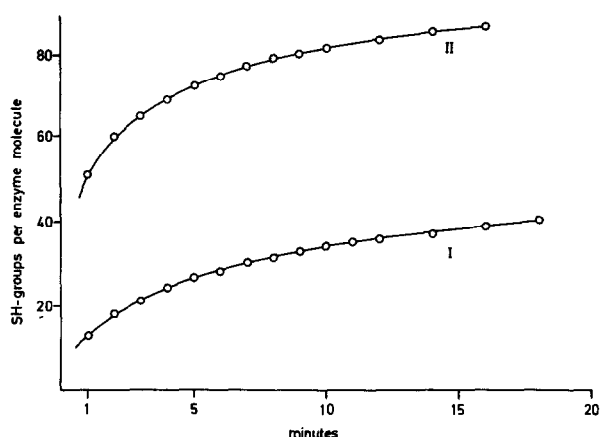


Fig. 3. Reaction of thiol groups in synthetase with DTNB. 2.25 mg/ml protein; 2×10^{-3} M DTNB.

(I) Fatty acid synthetase in 1 M NaCl.
(II) Same preparation after two freezings and thawings.

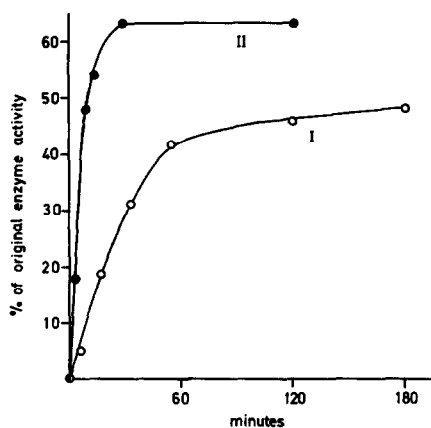
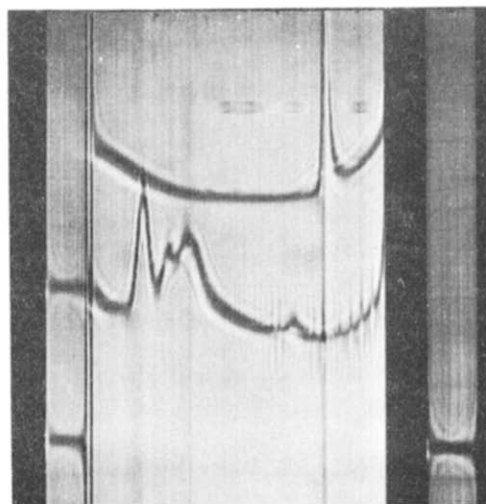


Fig. 4. Dependence of reactivation on time. Details of the procedure are reported in the experimental section.

(I) Fatty acid synthetase activity.
(II) β -Ketoacid reductase activity.

zyme. After a single freezing and thawing the appearance of more slowly sedimenting species can be noted. After two freezings native fatty acid synthetase is no longer present and enzymatic activity has vanished. The dissociation of the complex can be followed in a similar manner by analytical disc-electrophoresis. Fig. 2A presents the electrophoretic pattern of native fatty acid synthetase dissolved in 1 M LiCl; fig. 2B shows the same sample after freezing and thawing two times. The patterns indicate that the multienzyme com-

A



B

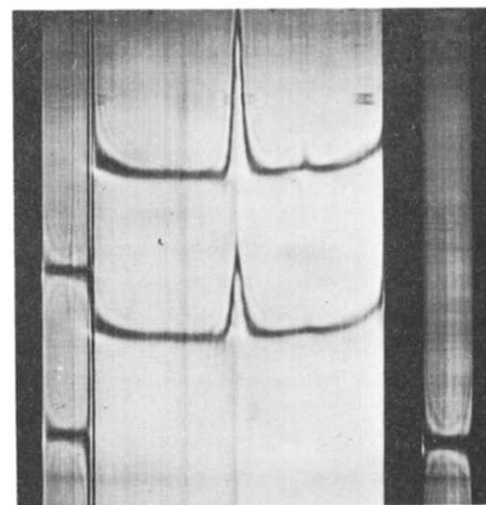


Fig. 5. Sedimentation behaviour of reconstituted fatty acid synthetase.

(A) and (B) (upper): Native fatty acid synthetase as reference under identical sedimentation conditions.

(A) (lower): Fatty acid synthetase (10 mg/ml) in 1 M NaCl after two freezings and thawings. Photograph taken 16 min after reaching the maximum speed of 59 780 rpm. 20°C.

(B) (lower): Same preparation as used in (A) after reactivation by dilution. 2 mg/ml protein; 0.1 M phosphate buffer pH 7.5. Photograph taken 6 min after reaching maximum speed of 59 720 rpm. 20°C.

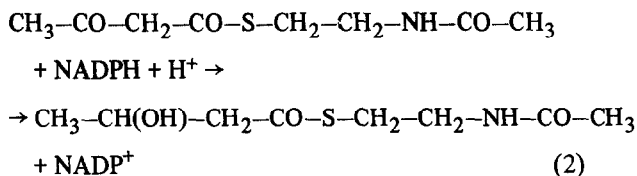
Sedimentation from left to right.

plex has been split into subunits.

The measurement of thiol group reactivity provides further evidence for structural changes in the

complex. Thiols react with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) with release of the deep yellow thiophenolate anion ($\lambda_{\text{max}} = 413 \text{ m}\mu$) which can be determined optically [9]. Amino acid analysis of the fatty acid synthetase showed that the complex contains 140 cysteine molecules [10]. All or almost all of these exist in the thiol form as shown by the optical method after denaturation of the fatty acid synthetase. If the synthetase is not denatured, however, only 20% of these thiol groups react within five minutes as shown in curve I of fig. 3. Reaction with the remainder of the thiol groups is probably hindered or prevented by the tertiary or quaternary structure of the complex. During the dissociation of the multi-enzyme complex these partially buried thiol groups should be exposed and a higher number of reactive thiols would be expected. Curve II of fig. 3 confirms this prediction. It represents reaction kinetics of the same enzyme preparation after two freezings and thawings.

Changes in fatty acid synthetase activity were followed optically. The synthesis depends on the proper functioning of all component enzymes. However, a loss of total synthetic activity does not imply loss of all partial reactions. The partial reactions can be measured independently with model substrates. To date we have confined ourselves to study the reduction step leading from β -ketoacid to β -hydroxyacid (first reduction), which is measured optically according to eq. (2):



After two freezings and thawings of a fatty acid synthetase solution 1 M in NaCl or LiCl, both the total synthetase activity and the reductase activity have completely disappeared. Reactivation occurs on decrease of ionic strength by dilution or dialysis. Fig. 4 shows the kinetics of reactivation of both activities. The partial activity reappears more quickly than total synthetase activity, which reaches 50% of the original value.

The reactivation process is strongly temperature-dependent and has an optimum at 20°C. At 0°C scarcely any reactivation occurs while at 37°C an initial fast

reactivation turns after 90 min again in the direction of decreased activity. The protein concentration also is influential. With concentrations lower than 0.5 mg/ml reactivation is not extensive. The addition of thiol compounds such as cysteine assists the process.

To compare the physical properties of native and reconstituted enzyme sedimentation and electrophoresis studies were undertaken. Fig. 5A shows the sedimentation pattern of a dissociated and a native enzyme under identical sedimentation conditions. Fig. 5B shows recombination in the treated sample after dilution. The dissociated sample of fig. 2B was likewise reactivated by dilution and subjected again to disc-electrophoresis (fig. 2C). Neither disc-electrophoresis nor analytical ultracentrifugation reveal any difference between native and dissociated enzyme.

4. Discussion

Freezing in salt solutions has been shown to cause dissociation or denaturation of a number of enzymes [11]. Although the nature of this process is not clearly understood, a "concentration effect" is assumed [12]. The liquid regions of the frozen system contain a high concentration of salt which changes such physical parameters as ionic strength, pH and water structure and leads to denaturation or dissociation.

The spontaneous recombination of subunits to form simple enzymes has been reported many times. Examples of enzymes composed of identical and non-identical subunits are known. Thiolase, for example, is built from four identical subunits [13]; aldolase from 2 α and 2 β subunits [14]. The aggregation of three different subunits to form pyruvate dehydrogenase represents an example of the spontaneous recombination at a much higher organizational level [15]. The construction of fatty acid synthetase moreover requires the proper orientation of six different enzymes and "acyl carrier protein" [16]. Apparently again in this case spontaneous aggregation occurs. An estimation of the molecular weight of the protein(s) in the most prominent of the electrophoresis bands (fig. 2B) according to the method of Martin and Ames [17] lead to values between 200 000 and 250 000. It is therefore questionable whether the described dissociation results in single enzyme components.

After dissociation of the complex it was surprising to find that β -ketoacid reductase activity had sunk to zero. Two explanations are conceivable. Either the native structure of the partial enzyme was altered by freezing and must first reactivate itself before association with neighbour enzymes is possible, or possibly the partial activity itself depends on protein-protein interactions with several neighbouring enzymes which become physically separated during dissociation. Further experiments can clarify this problem.

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