CRYSTALLIZATION OF A MULTIENZYME COMPLEX: FATTY ACID SYNTHETASE FROM YEAST

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Communicated June 10, 1969

Abstract.—The crystallization of purified fatty acid synthetase from yeast is facilitated by seeding techniques. The first seed crystals appeared as particles with almost negligible enzymatic activity in a solution that had been left at 4°C for 15 months. Subsequently, the crystallization time has been reduced to two days, and the crystals now isolated have retained their full enzymatic activity. The loss of synthetase activity that can result from prolonged exposure during crystallization is attributable specifically to damage of the enzyme component responsible for condensation activity. Crystalline synthetase is identical to purified but noncrystalline synthetase by all criteria so far examined.

Fatty acid synthetase has been isolated from yeast as a multienzyme complex with a molecular weight of 2.3×10^6 and outer dimensions of 210×250 Å, as determined by electron microscopy.[1, 2] In the presence of NADPH it catalyzes the conversion of acetyl- and malonyl-CoA into long-chain fatty acids according to the following over-all equation:

$$\begin{aligned} &\mathrm{CH_3{-}CO{-}SCoA} + \mathrm{n\ \overset{\overset{\mathrm{COOH}}{|}}{CH_2}{-}CO{-}SCoA} + \mathrm{2\ n\ NADPH} + \mathrm{2\ n\ H^+} \rightarrow \\ &\mathrm{CH_3{-}(CH_2{-}CH_2)_n{-}CO{-}SCoA} + \mathrm{n\ CO_2} + \mathrm{n\ CoASH} + \mathrm{n\ H_2O} + \mathrm{2\ n\ NADP^+}. \end{aligned}$$

At least six different enzymes in unknown stoichiometry are involved in catalysis of partial reactions: two transferases, a condensing enzyme, two reductases, and a dehydrase.[3] In addition to the proteins important for catalysis, another protein plays a role in substrate binding similar to that of "acyl carrier protein (ACP)," isolated from *E. coli* by Vagelos.[4] This protein accommodates a 4′-phosphopantetheine residue to which all acyl intermediates in fatty acid synthesis are bound in thioester linkage.[5–8] The 4′-phosphopantetheine forms a flexible arm of 20 Å which allows optimal juxtaposition of substrates and enzymes for the series of reactions which occur in the complex. A model of the synthetase has been proposed which places this flexible arm inside a space enclosed by the various enzymes.[9] Recent small-angle X-ray analysis confirms the existence of a hollow space in the rotationally symmetric enzyme complex.[10] Electron micrographs also give the impression of a cavity within the particle (Fig. 1). More extensive X-ray analysis would afford an elegant, if protracted, path toward precise knowledge of the synthetase's quarternary structure. This paper describes the crystallization of the complex prerequisite to studies of this type.

Materials and Methods.—Enzymes and coenzymes were purchased from Boehringer (Mannheim), serum albumin from Behring-Werke (Marburg), and glutaraldehyde from the Union Carbide Chemical Corp. Acetyl-CoA was prepared according to Simon and Shemin[11] by treatment of coenzyme A with acetic anhydride; malonyl-CoA was prepared

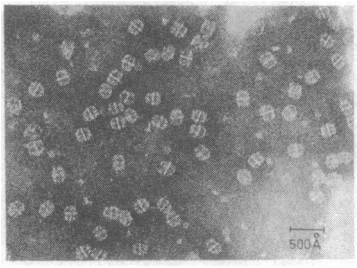

FIG. 1.—Electron micrograph of fatty acid synthetase. Fixation: 0.05 *M* sodium cacodylate + 2% glutaraldehyde; negative staining with potassium phosphotungstate pH 7.2; micrograph was taken by an Elmiskop I A, 80 kv; photo courtesy Dr. K. Rehn.

by transesterification from S-malonyl-N-capryloyl-cysteamine to coenzyme. A.[12] A Zeiss photomicroscope was used to take pictures of crystals.

Assay procedure: Protein was determined by the biuret method[13] using an Eppendorf spectrophotometer with a 546-mμ filter. The method was calibrated by dry weight determinations and led to the following formula for the protein contained in a 5-ml volume in a 2-cm cuvette: mg protein = OD × 8.61. The spectrophotometric method of Murphy and Kies[14] gives identical values, and the method of Warburg and Christian[15] gives proportional values higher by a factor of 1.25.

The activity of fatty acid synthetase was measured optically at 25°C by following the rate of NADPH oxidation at 334 mμ; 2 ml contained 200 μmoles potassium phosphate buffer pH 6.5, 20 μmoles cysteine, 0.6 mg serum albumin, 0.12 μmole acetyl-CoA, 0.3 μmole NADPH, and approximately 10 μg enzyme. After observation of the endogenous rate of NADPH oxidation for 3 min, the reaction was started by addition of 0.15 μmole of malonyl-CoA. An enzyme unit is defined as that amount of enzyme which catalyzes the incorporation of 1 μmole malonyl-CoA per minute into fatty acids. Partial reactions catalyzed by the complex were measured according to Lynen.[16]

Isolation of fatty acid synthetase: Details of the isolation procedure have been described by Lynen.[16] Yeast cells buffered in potassium phosphate pH 7.5 were disrupted by shaking with glass beads in a cell homogenizer constructed according to the specifications of Merkenschlager, Schlossmann, and Kurz.[17] The extract was subjected to ammonium sulfate fractionation, and the material precipitating between 35 and 50% saturation was collected, dialyzed, and adsorbed on calcium phosphate gel. Synthetase was selectively removed from the gel by elution with 0.067 *M* potassium phosphate pH 6.5. The ammonium sulfate fractionation was repeated, and the material precipitating between 35 and 50% saturation dissolved in 0.1 *M* potassium phosphate buffer pH 6.5 was centrifuged in a Spinco model L preparative ultracentrifuge at 100,000 *g* for 6 hr. The pellet was redissolved in buffer and insoluble material was separated by centrifugation at 37,000 *g*. The ultracentrifugation was then repeated to collect purified synthetase. Fatty acid synthetase prepared by this procedure possesses a specific activity of 1500–2500 mU/mg protein. Its sedimentation pattern in the analytical ultracentrifuge shows a single peak.

Crystallization: To a buffered solution of the enzyme, 3 *M* buffered ammonium sulfate was added slowly until there were signs of turbidity. The solution was then left for 2 hr. Afterwards water or 3 *M* ammonium sulfate solution was added as required until the solution strongly opalesced and appeared silky. After addition of seed crystals (*ca.* 1/500 of the total protein in solution), the solution was left at room temperature and the progress of crystallization followed with a microscope. In order to render the synthetase insoluble in water, the crystals were collected by centrifugation, resuspended in 1.5 *M* potassium phosphate buffer pH 5.5, and dialyzed for 12 hr against the same buffer. The buffer was changed 3 times during the course of the dialysis; 1–6 hr of dialysis against 1.5 *M* potassium phosphate pH 5.5 containing 0.1% glutaraldehyde followed, and finally dialysis against distilled water.

Measurement of the flavin content: Enzyme solutions containing 15–20 mg/ml protein were treated with 3 *M* trichloroacetic acid to a final concentration of 0.3 *M*. The supernatant obtained after centrifugation of denatured protein was neutralized with solid

potassium carbonate and the optical density at 445 mμ measured against a control containing no protein. The extinction coefficient used to calculate FMN concentrations was 12,200 $cm^2\ mmole^{-1}$.[18] Similar experiment with mixtures of FMN and serum albumin confirmed that this method for determining FMN concentrations was reliable.

Results—Conditions of crystallization The first crystals of yeast fatty acid synthetase were obtained only after a 0.9 per cent solution of the enzyme in 1.2 *M* ammonium sulfate at pH 6.5 had remained in a refrigerator at 0–4°C for 15 months. With the use of seed crystals, however, enzyme solutions now yield crystals within 4 to 6 weeks. The crystals under all conditions investigated are hexagonal prisms (Fig. 2). The only variation in crystals observed is the ratio of crystal length to crystal diameter, which changes from 1:5 when the mother liquor is at pH 7.5 to 1:1 at pH 5.5 (Fig. 3).

The largest crystals obtained to date have a length of 0.12 mm, but this length will certainly be exceeded in the future. Temperature has a strong influence on the speed of crystallization. At 4°C new crystals appear only after 4 to 6 weeks, while at 20°C new crystals are detectable after 10 to 13 days. At 30°C the rate

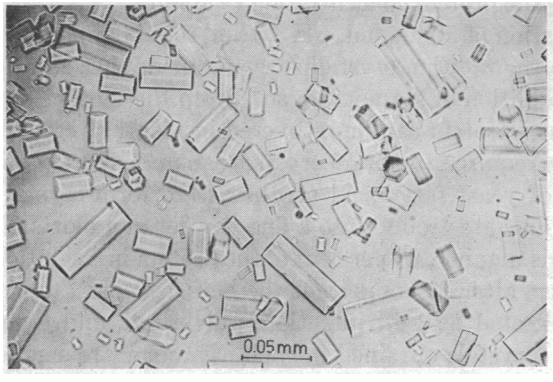

FIG. 2.—Crystals of fatty acid synthetase (pH 6.0).

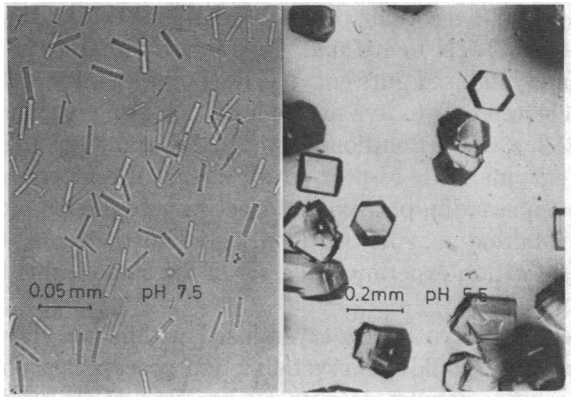

FIG. 3.—Crystals of fatty acid synthetase grown at different pH values.

TABLE 1. *Influence of protein concentration on the crystallization.**

Protein concentration (mg/ml)	Ammonium sulfate concentration (*M*)	Time of crystallization
72	0.95	2–3 days, ground seed crystals
49	0.92	10 days
42	0.87	14 days
34	0.82	After 14 days low yield, but large crystals
25	0.84	
16	0.97	
7	1.20	Microcrystalline or amorphous after 2 to 3 weeks
4	1.35	
1.2	1.50	

* At pH 5.5 and room temperature.

begins to decrease, and at 37°C no crystallization was observed. The influence of the pH is less pronounced. Of the pH values tested in the range from 5.5 to 8.0, the value 5.5 seems to be the best and simultaneously protects essential SH-groups from air-oxidation better than an alkaline pH. A third factor influencing crystallization is the protein concentration (Table 1).

At a concentration of 50 mg/ml, pH 5.5 and room temperature, 50–70 per cent of the protein in the solution crystallizes without further addition of ammonium sulfate. With addition of ammonium sulfate to the mother liquor, up to 90 per cent of the original protein can be recovered in identical crystal form. The unusually long time required to obtain crystals can undoubtedly be attributed to the weak tendency of yeast fatty acid synthetase to form crystal cores. For this reason, seed crystals are ground into a fine powder in a mortar from agate before addition. In this manner, 60 per cent of the protein in a solution can be obtained in crystalline form after 48 hours if, at the halfway point, the crystals that have appeared are collected by centrifugation, pulverized, and then re-added to the mother liquor. Crystals obtained in such a manner are smaller than those described above but possess the full enzymatic activity of the starting material. Because of the flavin component, the crystals appear yellow. Under the fluorescent microscope they emit a strong yellow fluorescence. The spectrum of a suspension of crystals, like a soluble preparation of synthetase, shows a shift in the 450-mμ band of free FMN to 464 mμ. The flavin content of the crystalline enzyme, like the uncrystallized pure enzyme, lies in the vicinity of 0.1 gm per 100 gm enzyme which corresponds to five molecules of FMN per synthetase unit of molecular weight 2.3×10^6. Solutions of washed enzyme crystals are identical to noncrystalline enzyme with respect to all chemical and enzymatic properties tested. The sedimentation pattern and electron microscope pictures of crystalline and noncrystalline enzyme are identical. Furthermore, the crystals and mother liquor are within experimental error of the same amino acid composition (Table 2).

Enzymatic activity: Synthetase crystallized within two days retains its full enzymatic activity. Should the crystallization process last one or two weeks, however, the specific activity sinks to 60 per cent of its original value. The activity of control solutions without seed crystals sinks to 22 per cent in the same period of time (Table 3).

TABLE 2. *Amino acid composition of fatty acid synthetase.*

Amino acid	Crystalline enzyme* (moles/2.3 × 10^6 gm)	Noncrystalline enzyme* (moles/2.3 × 10^6 gm)
Lysine	1356	1260
Histidine	332	326
Arginine	682	720
Aspartic acid	1974	2040
Threonine	1150	1160
Serine	1260	1240
Glutamic acid	2230	2280
Proline	921	920
Glycine	1346	1430
Alanine	1393	1470
Valine	1400	1335
Methionine	480	465
Isoleucine	1218	1160
Leucine	1662	1630
Tyrosine	588	582
Phenylalanine	840	818
Cysteine†	140	142
Tryptophan‡	410	398

* Various samples of protein were hydrolyzed for 24, 48, 72, and 97 hr; amino acid analysis was carried out with a Beckman Spinco amino acid analyzer, Model 120 B, and results were extrapolated to zero time hydrolysis.
† Determined as cystic acid.
‡ Determined spectrophotometrically.

TABLE 3. *Dependence of the enzymatic activity on the crystallization time.*

Days after addition of seed crystals	Specific activity of the enzyme* (mU/mg)	Specific activity of the control† (mU/mg)
0	1400	1390
4	1070	1070
7	690	505
11	800	290

* In a potassium phosphate pH 5.5 buffered solution with seed crystals.
† As above, but without seed crystals.

Incubation of deactivated crystals in a 0.01 *M* cysteine solution restores activity to 80 to 90 per cent of its initial value. The net loss of 10 to 20 per cent can be ascribed to damage of the condensing enzyme. Aged enzyme shows a decrease in synthetase activity proportional to the decrease in condensation activity.[19] This proportionality is understandable in view of the fact that condensation is the rate-limiting step in fatty acid synthesis.[9] The first crystals obtained after 15 months had retained only 4–6 per cent of the original activity. When such preparations are used for measurement of the seven partial reactions, the results are similar to those presented in Table 4. Only the condensation reaction decreased in proportion to total activity in accord with its role as the rate-determining reaction. This fact demonstrates that the condensing enzyme is the most labile component of the enzyme complex. Parallel increases in condensing activity as the synthetase is reactivated by cysteine treatment support this conclusion.

Of particular interest is the successful preparation of water-insoluble crystals through removal of ammonium sulfate in the crystal suspension by dialysis first

TABLE 4. *Residual enzymatic activities of the first crystalline preparation of fatty acid synthetase.*

Enzymatic activity	Original activity (%)
Fatty acid synthesis	5
Malonyltransfer	100
Acetyltransfer	100
Palmityltransfer	106
Condensation	5
First reduction	66
Dehydration	86
Second reduction	99

against potassium phosphate, then against 0.1 per cent glutaraldehyde, and finally against water. The coalescence of molecules in such crystals is so complete that neither formic acid nor dilute ammonia can dissolve them. Only by pepsin treatment could the crystals be destroyed. The ability of the crystals to rotate the plane of polarized light, as demonstrated by polarization microscopy, was not lost after treatment with glutaraldehyde. This indicated that the internal structure of the crystals had not been altered. The synthetase activity of the crystals, however, was lost after 40-minute exposure to this reagent. Since crystals exposed for 60 minutes nonetheless retained 80 per cent of their β-keto-acid reductase activity, insolubility in water did not signify complete loss of all catalytic properties. This experiment is important for the transfer of crystals into an organic solvent containing polymerizable monomers. Electron deflection experiments with crystal slices would be a valuable extension of X-ray analysis.[20] Experiments of this nature are presently being carried out by the group of W. Hoppe.

The authors thank J. Ziegenhorn for measurement of partial activities shown in Table 4. Thanks are also extended to Dr. Th. Orme for help in the preparation of the manuscript.

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