Fatty Acid Synthetases from *Euglena gracilis*

SEPARATION OF COMPONENT ACTIVITIES OF THE ACP-DEPENDENT FATTY ACID SYNTHETASE AND PARTIAL PURIFICATION OF THE $\beta$-KETOACYL-ACP SYNTHETASE*

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The component enzymes of the chloroplast-associated, acyl carrier protein (ACP)-dependent, fatty acid synthetase (FAS-II) from *Euglena gracilis* have been independently examined by gel filtration chromatography of crude extracts from photoautotrophic cells. The acetyl coenzyme A:ACP transacylase, malonyl CoA:ACP transacylase, and $\beta$-ketoacyl-ACP reductase activities were clearly resolved with apparent molecular weights of 147,000, 106,000, and 44,000, respectively. The $\beta$-ketoacyl-ACP synthetase, $\beta$-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase activities in crude extracts migrated in an unresolved peak with an apparent molecular weight of 280,000. FAS-II activity, which exhibited an apparent molecular weight of 173,000, resulted from the overlapping portions of the six component activities.

Under certain conditions, several of the *E. gracilis* FAS-II component activities may aggregate noncovalently to form a weak complex. A partial purification of the $\beta$-ketoacyl-ACP synthetase by ammonium sulfate fractionation, DEAE-cellulose chromatography, and hydroxylapatite chromatography resulted in its complete separation from the enoyl-ACP reductase activity. A portion of the enoyl-ACP reductase activity co-purified with the $\beta$-ketoacyl-ACP synthetase activity through the DEAE-cellulose chromatography. When the separated $\beta$-ketoacyl-ACP synthetase and enoyl-ACP reductase activities were recombined and subjected to gel filtration chromatography, the two activities migrated distinctly and with lower apparent molecular weights, 118,000 and 56,500, respectively, than when similarly measured in the crude extract.

The alga *Euglena gracilis* contains two fatty acid synthetases which are controlled by a photoregulatory mechanism (1-4). Dark-grown cells of *E. gracilis* contain a cytoplasmic ACP-independent fatty acid synthetase multienzyme complex (FAS-I). When grown in the light, the cells contain, in addition to FAS-I, a chloroplast-localized ACP-dependent fatty acid synthetase (FAS-II).

Although ACP-dependent fatty acid synthetases have been described in several other bacteria, algae, and plants, the *Escherichia coli* system is the only one from which the component enzymes have been separated and purified (5). The component enzymes of ACP-dependent fatty acid synthetases have been generally assumed to occur as nonaggregating entities, but this assumption has not been tested directly. This report describes an investigation of possible physical interactions among the component enzymes of the *Euglena* chloroplast ACP-dependent fatty acid synthetase and a partial purification of the $\beta$-ketoacyl-ACP synthetase.

**MATERIALS AND METHODS**

**RESULTS AND DISCUSSION**

Independent Assays of Euglena FAS-II Component Activities—The NADH and NADPH requirements of FAS-II were tested after separation of FAS-II from FAS-I by ammonium sulfate fractionation as previously described (1). FAS-II was optimally active with NADPH alone, indicating that both the $\beta$-ketoacyl-ACP reductase and enoyl-reductase of FAS-II are specific for NADPH.

Assays with linear activity versus protein relationships were developed for each of the FAS-II component activities ("Materials and Methods").

The substrate specificities of the *Euglena* FAS-II enoyl-ACP reductase and $\beta$-ketoacyl-ACP reductase were studied with respect to thioester as well as reductant (Table I). The fastest rate of reduction of N-acetyl-S-acetoacetyl-ACP was found with a combination of NADH and NADPH. The important finding, however, was that acetoacetyl-coenzyme A was much more rapidly reduced than acetoacetyl-ACP by a combination of NADH and NADPH. In contrast, when NADPH was used as the sole reductant, acetoacetyl-ACP was readily reduced, but acetoacetyl-CoA was completely inactive. A substantial portion of the acetoacetyl-ACP reductase activity determined in the presence of both NADH and NADPH is likely due to $\beta$-hydroxyacyl-CoA dehydrogenase activity of the fatty acid $\beta$-oxidation pathway (16). The specific reduction of acetoacetyl-ACP can, therefore, be validly measured in *Euglena* crude extracts only when NADPH alone is used as the reductant.

The *Euglena* enoyl reductase substrate specificities shown in Table I are very similar to those reported for the purified enzymes. Portions of this paper (including "Materials and Methods" and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-866, cite author(s), and include a check or money order for $1.20 per set of photocopies. The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PPO, 2,5-diphenyloxazole; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; N-morpho1ino)ethanesulfonic acid; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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‡ The abbreviations used are: ACP, acyl carrier protein; FAS-I, ACP-independent fatty acid synthetase; FAS-II, ACP-dependent fatty acid synthetase; N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
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E. coli enoyl reductase, which is almost equally active with NADH or NADPH as reductant when crotonyl-ACP is the substrate and is quite specific for the ACP substrate (17).

The FAS-II condensing enzyme activity was dependent on the addition of N-acetyl-S-malonyl-ACP and was completely inhibited by 500 μM cerulenin. This antibiotic is a specific and irreversible inhibitor of the E. coli condensing enzyme (18).

Separation of the FAS-II Component Activities by Gel Filtration Chromatography—The possibility of physical association among some or all of the FAS-II component activities was investigated by gel filtration chromatography. A crude extract from photoautotrophic Euglena was fractionated on a Bio-Gel A-1.5m column. As shown in Fig. 1A, FAS-I activity was present in the void volume of the column and was completely separated from the FAS-II activity which eluted later. Only one activity peak was observed for each FAS-II component activity; therefore, FAS-I does not significantly interfere with any of the FAS-II component activity assays. The apparent peak of condensing enzyme activity (Fig. 1D) appearing in the void volume was shown to be an artifact since the "activity" was not dependent on N-S-acetyl-ACP or N-acetyl-S-malonyl-ACP. No ketoacyl reductase activity was found in any of the fractions, and it was assumed that this activity was destroyed by the freezing and thawing of the fractions. An ammonium sulfate fraction (35 to 70% of saturation) of a crude extract from photoautotrophic Euglena was then chromatographed over the same column, and the fractions were assayed immediately for ketoacyl reductase activity. The ketoacyl reductase activity peak, which was observed under these conditions, is shown in Fig. 1E.

The FAS-II activity peak coincides with the overlapping portions of the separate FAS-II component activity peaks. Three of the component activities, condensing enzyme, enoyl reductase, and dehydrase, were not significantly separated from one another. The remaining three activities, acetyl transacylase, malonyl transacylase, and ketoacyl reductase, were each partially but clearly separated from the remaining activities. Euglena FAS-II is, therefore, not a tight multi-enzyme complex lacking only ACP.

The peak containing the three unseparated activities is significantly wider than the other peaks, and the ketoacyl reductase peak exhibits a definite shoulder positioned exactly in the region of overlap with the remaining FAS-II component activities. Similarly, the acetyl transacylase and malonyl transacylase activities from E. coli were observed in an early report to overlap partially on gel filtration, with each peak containing a significant shoulder (19).

The Bio-Gel A-1.5m column used for the separation of the FAS-II component activities was calibrated with standard proteins of known molecular weight. The apparent molecular weights of the FAS-II components determined from the elution volumes in Fig. 1 are 147,000, 106,000, 322,000, 4,400, 2,81,000, and 240,000 for acetyl transacylase, malonyl transacylase, FIG. 1. Gel filtration chromatographic separation of FAS-I and component activities of FAS-II. A crude extract (1.5 ml) from photoautotrophic Euglena (34 mg of protein) was chromatographed on a Bio-Gel A-1.5m column (80.4 ml) as described under "Materials and Methods." Samples (10 μl) of each fraction were assayed for FAS-I and the component activities of FAS-II as described under "Materials and Methods." A. fatty acid synthesis in the presence (○–○) and absence (■–■) of ACP. A280 (■–■), B. condensing enzyme assayed by the increase in A340 with acetyl-ACP and malonyl-ACP (○–○), with acetyl-ACP alone (△–△), or in the absence of both acetyl-ACP and malonyl-ACP (■–■), C. 1.0 ml of an ammonium sulfate fraction (35 to 70% of saturation) of a crude extract from photoautotrophic Euglena (36 mg of protein) was chromatographed exactly as described above; β-ketoacyl-ACP reductase (○–○), A280 (■–■).
acylase, condensing enzyme, ketoacyl reductase, dehydrase, and enoyl reductase, respectively. These apparent molecular weight values will reflect any aggregation which may occur between the FAS-II components and other components of the crude extract and may, therefore, not coincide with values determined on purified components (see below). The characterized *E. coli* FAS-II enzymes range in molecular weight from 35,500 to 170,000 (14, 17, 20, 21).

An experiment similar to that described in the legend to Fig. 1, i.e., one in which the apparent molecular weights of the component activities are determined under conditions where aggregation among the activities might be detected, has never been reported for the *E. coli* fatty acid synthetase.

Separation of Euglena Condensing Enzyme and Enoyl Reductase—As shown in Fig. 1, the *Euglena* condensing enzyme and enoyl reductase activities overlapped almost completely on gel filtration chromatography of a crude extract, and the apparent molecular weight of the condensing enzyme was quite large (>5 times) in comparison with that of the *E. coli* condensing enzyme. An investigation of the possible association between these two activities was made in connection with a partial purification of the *Euglena* condensing enzyme activity.

A crude extract from photoautotrophic *Euglena* was treated with streptomycin sulfate, fractionated with ammonium sulfate, and applied to a DEAE-cellulose column which was then eluted with a LiCl gradient. As shown in Fig. 2, the condensing enzyme and enoyl reductase activities eluted at virtually the same point, near a conductivity of 6 millisiemens. Although different absolute quantities of the condensing enzyme and enoyl reductase were originally present in the crude extract, almost equal amounts, i.e., numbers of units, of the two activities were recovered in the peak from the DEAE-cellulose column.

The fractions from a DEAE-cellulose column containing condensing enzyme activity were pooled and applied directly to a hydroxylapatite column which was then eluted with a potassium phosphate gradient. The condensing enzyme activity was eluted as a single peak at a conductivity of 15.4 millisiemens and was completely separated from the enoyl reductase activity which was eluted as a single peak at a conductivity of 6.5 millisiemens.

Details of the partial purification of the *Euglena* condensing enzyme are summarized in Table II. A 60-fold net purification was achieved with a 49% yield.

The co-migration of the condensing enzyme and enoyl reductase activities on a gel filtration column (Fig. 1) and a DEAE-cellulose column (Fig. 2), although possibly fortuitous, suggests that under these conditions a portion of the activities existed as a noncovalent complex which was disrupted on hydroxylapatite.

The capacity of the separated condensing enzyme and enoyl reductase to associate when recombined was tested by mixing fractions containing the two activities obtained from the hydroxylapatite column and rechromatographing the mixture on the same Bio-Gel A-1.5m column used to obtain the profiles shown in Fig. 1. As shown in Fig. 3, the recombined condensing enzyme and enoyl reductase activities were clearly separated on the Bio-Gel A-1.5m column. Moreover, the molecular weights obtained from Fig. 3, 118,000 for the condensing enzyme and 56,500 for the enoyl reductase, are much lower than the values obtained when the crude extract was chromatographed on the same column.

These results do not rule out the possibility that the separated condensing enzyme and enoyl reductase activities can reassociate. For example, the relative and absolute concentrations of the two activities in the recombined fractions differed from those in the crude extract, and the presence or absence of other factors in the crude extract could influence the association of the FAS-II components.

The *E. coli* fatty acid synthetase and now the *E. gracilis* FAS-II system are the only fatty acid synthetase systems which have been shown to consist of separable component activities. The chloroplast fatty acid synthetases from spinach (22), lettuce (22, 23), avocado mesocarp (24, 25), and *Chlamydomonas reinhardii* (26) are all dependent on ACP and presumably consist of separable component activities also, but this has not been tested.

REFERENCES

Euglena gracilis Fatty Acid Synthetase Components

Fatty Acid Synthetases from Euglena gracilis. Separation of the component activities of the &alpha;-&beta; subunits, and partial purification of the &alpha; subunit of the synthesizing enzyme.

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Materials and Methods

NADPH, NADH, acetyl-CoA, malonyl-CoA, acetoacetyl-CoA, and acetoacetoacetyl-CoA were purchased from Sigma Chemical Co., St. Louis, Mo. Acetate, &beta;-methylacetate, propionate, and malate were purchased from New England Nuclear. [14C]NADH and [1-14C]acetate were purchased from New England Nuclear. [3H]NADPH and [1-3H]acetate were supplied by W. M. Stolz. [2-14C]Acetate and [1-14C]malate were purchased from Amersham Radiochemicals. NADH was obtained from Sigma Chemical Co. and NADPH from Fluka Chemicals. Acetoacetate, malonyl-CoA, and propionyl-CoA were supplied by Sigma Chemical Co. Acetoacetoacetyl-CoA was prepared from 3-hydroxy-3-methylglutaryl-CoA and propionyl-CoA.

Methods

Electrophoresis of extracts was carried out in a 6% polyacrylamide gel and was as described by Nisole. The enzymes were solubilized in 2% sodium dodecyl sulfate, 50 mM sodium phosphate buffer, pH 7.5, containing 20% glycerol and 0.01% bromophenol blue. After electrophoresis, the gel was sliced into 3-mm-thick pieces and immersed in 0.5 ml of 1 M NaOH. After 18 h, the activity was determined as described by Nisole.

Results

The initial activity of the &alpha; subunit was measured as the rate of incorporation of [1-14C]acetate into malonyl-CoA. The enzyme was solubilized in 2% sodium dodecyl sulfate, 50 mM sodium phosphate buffer, pH 7.5, containing 20% glycerol and 0.01% bromophenol blue. After electrophoresis, the gel was sliced into 3-mm-thick pieces and immersed in 0.5 ml of 1 M NaOH. After 18 h, the activity was determined as described by Nisole.

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Conclusion

The results presented here demonstrate that the Euglena gracilis fatty acid synthetase is composed of two distinct subunits, &alpha; and &beta; subunits, which are encoded by separate genes. The &alpha; subunit is the enzyme that catalyzes the condensation of acetyl-CoA and malonyl-CoA to form palmitoyl-CoA. The &beta; subunit is the enzyme that catalyzes the chain elongation by the condensation of malonyl-CoA and the previous fatty acyl-CoA to form palmitoleoyl-CoA. These results provide new insights into the molecular biology of fatty acid synthesis in Euglena and other photosynthetic eukaryotes.
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**Preparation of Enzyme Containing Organelle (Golgi Complex) and Membrane Fraction**

A 1.4% (v/v) solution of Glucose-6-Phosphate Dehydrogenase (G6PDH) in 0.1 M sodium phosphate buffer, pH 7.5, was incubated at 37°C for 30 min. The reaction was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 10%.

**Preparation of Membrane Fraction**

A 1.4% (v/v) solution of Glucose-6-Phosphate Dehydrogenase (G6PDH) in 0.1 M sodium phosphate buffer, pH 7.5, was incubated at 37°C for 30 min. The reaction was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 10%.

**Preparation of Golgi Enriched Fraction**

A 1.4% (v/v) solution of Glucose-6-Phosphate Dehydrogenase (G6PDH) in 0.1 M sodium phosphate buffer, pH 7.5, was incubated at 37°C for 30 min. The reaction was terminated by the addition of trichloroacetic acid (TCA) to a final concentration of 10%.

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Fatty acid synthetases from Euglena gracilis. Separation of component activities of the ACP-dependent fatty acid synthetase and partial purification of the beta-ketoacyl-ACP synthetase.

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