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Fatty Acid Synthetase Activity in *Euglena gracilis* Variety *bacillarius*. Characterization of an Acyl Carrier Protein Dependent System†

Mary Lou Ernst-Fonberg

ABSTRACT: *Euglena gracilis* variety *bacillarius* has been shown to have two fatty acid synthetase systems which differ in organizational complexity. One enzyme system is independent of added acyl carrier protein (ACP) for activity *in vitro* and is a complex of molecular weight greater than 1,000,000. The second synthetase is dependent on added ACP for activity. Bio-Gel chromatography of the latter system suggests that it is associated loosely in an active complex with a molecular weight of about 360,000. Studies of the kinetics of the ACP-dependent system gave parameters which are similar to those reported for multienzyme complex fatty acid synthetases which contain tightly bound ACP. Investigation of ACP substrate dependency showed that when ACP and the enzymes are incubated together prior to initiation of reaction a sigmoidal relationship between ACP and initial reaction velocity and the highest V_{max} was obtained. The degree of expression of each of the two fatty acid synthetases in comprising total enzyme activity is dependent on the stage of chloroplast development.

Studies on fatty acid biosynthesis with preparations from a variety of organisms have indicated that there are two types of synthetase systems which differ in organizational complexity. In yeast (Lynen, 1961) and animals (Burton *et al.*, 1968; Hsu and Yun, 1970; Larrabee *et al.*, 1965; Smith and Abraham, 1970) the steps of fatty acid biosynthesis from malonyl-CoA and acetyl-CoA are catalyzed by a multienzyme complex containing tightly bound ACP.[1] In plants (Overath and Stumpf, 1964; Brooks and Stumpf, 1966; Simoni *et al.*, 1967) and bacteria (Alberts *et al.*, 1963; Goldman *et al.*, 1963; Lennarz *et al.*, 1962) except mycobacteria (Brindley *et al.*, 1969), a notable exception which represents the more advanced procaryotes, the overall reaction of fatty acid biosynthesis is carried out by a series of reactions catalyzed by discrete enzymes which are not isolated as multienzyme complexes. In the latter systems, ACP is easily separated from the other proteins involved in the reactions.

Euglena gracilis is particularly interesting because it exhibits characteristics of both plants and animals. Strain Z of this organism was found by Delo *et al.* (1971) to possess both types of fatty acid synthetase systems. Further study (Ernst-Fonberg and Bloch, 1971) indicated that the ACP-dependent fatty acid synthetase present in the organism is linked with chloroplast development and the chloroplast protein biosynthetic apparatus. These studies have been extended to *Euglena gracilis* variety *bacillarius* with the hope of using several chloroplast mutants of this variety to define better the role of the organelle in the appearance of the ACP-dependent fatty acid synthetase. The results of this work will be reported shortly. Here is presented a description of the fatty acid syn-

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[1] Abbreviations used are: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; ACP, acyl carrier protein; NADPH, reduced nicotine adenine dinucleotide phosphate.

thetase enzymology in *Euglena gracilis* variety *bacillarius* and a partial characterization of the ACP-dependent enzyme system.

Experimental Section

Materials

Ammonium sulfate, grade I, purified, and NADPH were obtained from Sigma Chemical Co. Tes buffer and dithiothreitol were purchased from Calbiochem. Fatty acids, malonyl-CoA, and acetyl-CoA were obtained from P-L Biochemicals. New England Nuclear was the source of [2-^{14}C]malonyl-CoA and [2-^{3}H]acetyl-CoA. *Escherichia coli* ACP was purified according to the procedure of Majerus *et al.* (1964). Bio-Gel A-15 was obtained from Bio-Rad Laboratories. Buffer I was a pH 7.0, 0.01 M potassium phosphate buffer which was 0.002 M in dithiothreitol.

Methods

Growth of Cells. Euglena gracilis variety *bacillarius* were a generous gift from Professor J. A. Schiff of Brandeis University, Waltham, Mass. The cells were grown in 1 l. of Hutner's "High Yield" heterotrophic medium (Wolken, 1967) in 2-l. flasks with constant rotary shaking in the dark. The room was equipped with green safe lights (Withrow and Price, 1957). In experiments where green cells were required, the etiolated cells were harvested under sterile conditions and resuspended in resting medium (Stern *et al.*, 1964). Following a period of 48 hr in the dark to ensure the completion of any cell division in progress, the cultures were exposed to light, 150 ft-candles, for 24 hr and then harvested. In all instances, cultures at the final harvest were checked for contamination by monitoring with phase contrast microscopy and by plating an aliquot on pH 6.8 nutrient agar.

Enzyme Assays. Two assay procedures were used to measure the activities of the fatty acid synthetases. One assay measured the incorporation of [2-^{14}C]malonate into long-chain fatty acids (Delo *et al.*, 1971). *E. coli* ACP 5.65–9.05 $\times$ 10^{-6} M was added to the reaction when measurements of the ACP-dependent enzyme system were made. In this assay, 1 unit of enzyme activity was defined as that amount which catalyzed the incorporation of 1 nmol of [2-^{14}C]malonate into fatty acids in 15 min. The second assay was a spectrophotometric measure of the rate of oxidation of NADPH. The solution included: malonyl-CoA, 15 nmol; acetyl-CoA, 7.5 nmol; NADPH, 24 nmol; ACP, 20 μg. It was 0.1 M in Tes buffer (pH 7.2) and 0.002 M in dithiothreitol. The final volume was adjusted to 0.25 ml with water. Following a 10-min incubation period of all components, the reaction was initiated, and the decrease in absorbance at 338 nm was monitored on a Gilford 240 equipped with a Gilford 6040 recorder and a Haake circulating water bath. A decrease in absorbance of 0.012 unit/min was defined as 1 unit of enzyme activity. In the reaction volume employed, this decrease in absorbance indicated the oxidation of 0.5 nmol of NADPH.

Preparation of Enzyme Extracts. The cells (1–4 g) were suspended in four times their volume of buffer I at 0–4°. When quantitation of enzyme activity for comparative purposes was desired, the cell suspension was brought to a known volume and an aliquot was taken for dry weight determination. The mixture was purged with N_2; then the cells were disrupted with ultrasound, three bursts of 30 sec each at an average of 75 W. A Branson Model S 125 Sonifier was used. Cell breakage was checked microscopically. Following disruption, the mixture was purged briefly with N_2 then centrifuged at 49,000*g* for 30 min. The supernatant was brought to 70% saturation with ammonium sulfate at 4° by the addition of the appropriate volume of a solution of buffer I saturated with ammonium sulfate. After 30 min, the precipitate which contained both fatty acid synthetase systems was collected and dissolved in 2 ml of buffer I and stored below −20°.

The two fatty acid synthetase systems were separated by molecular sieve chromatography. A column, 2.5 $\times$ 23 cm of Bio-Gel A-15 equilibrated and eluted with buffer I, was prepared and run at 4°. The column was calibrated with a mixture of Blue Dextran, ferritin, bovine globulin, and catalase applied in a volume of 2 ml. The same volume of a 0–70% saturation ammonium sulfate fraction from 3 g of greened cells was chromatographed. Fractions of 4 ml were collected and assayed for fatty acid synthetase activity and absorbance at 280 nm. Similar experiments were carried out on ammonium sulfate fractions prepared from extracts of etiolated cells.

In all column chromatographic experiments, fractions included under distinct peaks of fatty acid synthetase activity were pooled and immediately brought to 70% saturation with ammonium sulfate. The precipitated enzymes were collected and dissolved in 1.5 ml of buffer I. This material was stored below −20° and later analyzed for enzyme activity in the presence and absence of ACP. These partially purified fatty acid synthetases were used in all studies of the two enzyme systems.

In several instances, the pooled and precipitated fractions containing ACP-dependent enzyme activity were rechromatographed under the same conditions. Fractions in both areas of potential enzyme activity were assayed. The portion of the chromatogram containing the ACP-dependent activity was again pooled, precipitated, and measured for enzyme activity. Protein was determined by the method of Lowry *et al.* (1951) as modified by Eggstein and Kreutz (1955).

Product Identification. Thin-layer chromatography on Eastman Chromogram silica gel sheet 6061 was used to identify fatty acids as a general class. The solvent system was hexane–ethyl ether–acetic acid (80:20:1, v/v) described in Eastman Chromogram sheet V-21. The standards were detected by spraying with a 2% solution in ethyl alcohol of 2′,7′-dichlorofluorescein. The radioactive products of the enzyme reactions were isolated by cutting the chromatogram serially into strips 1 $\times$ 4 cm, placing them in 15 ml of toluene–2,5-diphenyloxazole and measuring the radioactivity. Quenching was monitored by the channels ratio method.

The chain length of the fatty acid product was estimated by carrying out the biosynthesis in the presence of [2-^{3}H]acetyl-CoA and [2-^{14}C]malonyl-CoA. The amount of each radioisotope incorporated into product was determined, and the ratio of ^{14}C to ^{3}H was used as an estimate of the chain length of the fatty acid formed. The ^{3}H served as a marker of the methyl end of the molecule while ^{14}C was a measure of 2-carbon units added to the growing fatty acid chain.

An attempt was made to determine if the products of the reaction catalyzed by the ACP-dependent fatty acid synthetase were free or ACP-bound fatty acids. A two-layered column was prepared with Bio-Gel P-2, 2.5 $\times$ 8.7 cm on the bottom, and Bio-Gel P-100, 2.5 $\times$ 10.3 cm on top. A disk of filter paper separated the two layers. The column was equilibrated and eluted with 0.5 M acetate buffer (pH 5.0). An approximate calibration was made by chromatographing 0.5 ml of a solution containing ferritin, cytochrome *c*, bacitracin, dichlorofluorescein, and tyrosine. Triplicate samples of a radioactive enzyme assay of the ACP-dependent fatty acid synthetase were prepared. At the end of the reaction incubation period,

two samples were processed to completion of the assay, and the other sample was applied immediately to the column. The column was run at room temperature at a flow rate of 7.75 ml/cm^2 per hr. The 1-ml fractions were assayed for radioactivity by adding a 0.1-ml aliquot to 15 ml of toluene–2,5-diphenyloxazole and 3 ml of ethanol.

The nature of the product formed by the ACP-dependent system was also determined by measuring the amount of fatty acids extracted from an acidified assay solution before and after saponification. The assay measuring the incorporation of [2-^{14}C]malonyl-CoA into pentane-extractable product was done as described. Immediately at the end of the reaction period, the assay tubes were placed on ice and brought to pH 1.0 with 0.05 ml of concentrated HCl. The solutions were extracted three times with 5-ml portions of pentane. The extracts were combined and evaporated, and the radioactivity was measured. The assay solution was made alkaline with 0.2 ml of 45% KOH and heated at 100° for 30 min. The solutions were again acidified, extracted, and measured as before.

Results

Fatty Acid Synthetase Present in Etiolated and Greened Cells. Chromatography of the ammonium sulfate fractionated greened cell extract on Bio-Gel A-15 showed two discrete peaks of fatty acid synthetase activity (Figure 1). The enzymic activity that emerged with the first peak was fully active and independent of added ACP, whereas the activity that emerged later required added ACP for full expression. Chromatography of similar material from etiolated cells gave a chromatogram with only one peak of enzyme activity in the area of fractions 12–17.

Similar experiments were done on materials obtained from measured aliquots of a single culture, a portion of which had been kept in the dark while the other part was exposed to light for 24 hr. Following chromatography, fractions within the two peak areas were pooled, precipitated, and analyzed for enzyme activity. The activities were normalized to the dry cell weight of a measured aliquot of each of the two portions of cells; the relative enzyme activities are shown in Table I. The total synthetase activity is similar from greened and etiolated cells; however, the enzymic sources of the activity vary. In the etiolated cells, only a small portion of the total fatty acid synthetase activity stems from the ACP-dependent enzyme system. Following exposure of the cells to light, the activity of the ACP-independent multienzyme complex is about one half of that obtained from etiolated cells while the ACP-dependent enzyme system accounts for 53% of the total fatty acid synthetase activity. Clearly, the change in the developmental phase of the organism is reflected in changes in both of the fatty acid synthetase enzyme systems.

Characterization of the ACP-Independent Enzyme System. Localization of this enzyme activity in the effluent of a calibrated Bio-Gel column gave a molecular weight estimation of about 1.5 million. The substrate requirements of the partially purified multienzyme complex include NADPH, malonyl-CoA, and acetyl-CoA.

The effect of enzyme concentration on the rate of product formation showed that the reaction was linear at low enzyme concentrations and declined at higher concentrations of enzyme.[2] All measurements of activity were made in the linear region.

[2] This observation was made initially in this laboratory by Mrs. Zdenka Jonak. The ACP-independent enzyme system has been purified extensively and will be the subject of a separate report.

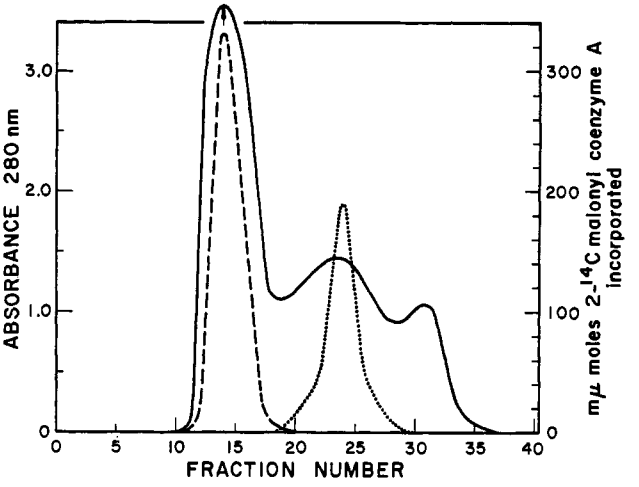

FIGURE 1: Separation of ACP-dependent and ACP-independent fatty acid synthetases on Bio-Gel A-15. A 0–70% ammonium sulfate fraction obtained from greened cells was applied to a 2.5 × 23 cm column prepared in and eluted with buffer I. Fractions of 4 ml were collected and measured for absorbance at 280 nm (—) and fatty acid synthetase activity as described in the text with (· · · ·) and without (- - - -) ACP. The first protein peak eluting was totally independent of ACP for full activity. The second activity peak was dependent on ACP.

Characterization of the ACP-Dependent Enzyme System. The ACP-dependent system which consists of at least six separate enzymes has been obtained in a partially purified form. Further attempts at purification have been deferred until some characterization of the entire system of enzymes could be made before separating the system into component enzymes with further purification. During the course of the described preparation, the ACP-dependent enzyme system was purified about 5-fold.

The ACP-dependent fatty acid synthetase activity chromatographed on the calibrated Bio-Gel column in an area corresponding to a molecular weight of 360,000. When the fractions (20–26) comprising the peak of ACP-dependent activity (Figure 1) were pooled, precipitated, and assayed, the activity was much greater than the sum of that measured in the individual fractions (Table II). This observation obtained on rechromatography of the chromatographed material indicating that the initial observation was not due to the removal of an inhibitor by the ammonium sulfate precipitation. Fol-

TABLE I: Composition of Fatty Acid Synthetase Activities in Etiolated and Greened *Euglena*.[a]

Source of Enzymes	Total ACP-Dependent Act.	Total ACP-Independent Act.	Total Fatty Acid Synthetase Act.	% ACP Dependency
Etiolated cells	83	1914	1996	4
Greened cells	1044	913	1957	53

[a] The ACP-dependent and ACP-independent enzyme systems were separated on a Bio-Gel A-15 column. The separated activities were pooled, concentrated by ammonium sulfate precipitation, and assayed for fatty acid synthetase activity as incorporation of [2-^{14}C]malonate as described. An ACP concentration of 5.65×10^{-6} M was used in these experiments.

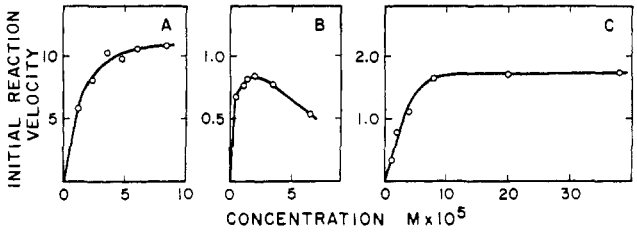

FIGURE 2: Relationship between initial reaction velocity and substrate concentration in the ACP-dependent enzyme system. (A) Malonyl-CoA substrate dependency. The initial reaction velocity was measured spectrophotometrically as described in the Methods section except that the amounts of malonyl-CoA were varied as shown. (B) Acetyl-CoA substrate dependency. The initial reaction velocity was determined by measuring the incorporation of [2-^{14}C]-malonate into fatty acids (Delo *et al.*, 1971). Components of the reaction were incubated at 35° for 5 min prior to initiation of reaction. The reaction solution included: ACP, 9.05 μM; NADPH, 90 μM; [2-^{14}C]malonyl-CoA (specific activity 0.5), 60 μM; and acetyl-CoA as shown. It was 0.1 M in Tes buffer (pH 7.2) and 0.002 M in dithiothreitol in a final volume of 0.5 ml. (C) NADPH substrate dependency. The initial reaction velocities were determined as in B except that acetyl-CoA, 30 μM, was used, and NADPH concentration was varied as shown. In all instances, reactions were initiated by the addition of malonyl-CoA.

lowing the initial chromatography of enzyme obtained from greened cells, the total activity summed from fractions 20–26 was 299 units. Upon collection and concentration, the total activity was 990 units; 99% of the enzyme activity was dependent on added ACP. Rechromatography of the pooled and concentrated material from fractions 20 to 26 (990 units of enzyme activity) showed practically no peak of enzyme activity in this area (14 units of activity). As shown in Table II, the total activity in these fractions increased markedly upon pooling and concentrating (347 units of enzyme activity). A 35% recovery of activity was obtained from this area relative to the first chromatography; this is a reasonable recovery since the enzyme system tends to lose activity at 4°, the temperature at which chromatography was done. Identical results were obtained when the activity of the pooled fractions was measured rather than summing the activity of the individual fractions. These experiments are compatible with the likelihood that the ACP-dependent enzyme system is a series of discrete enzymes which may need to be loosely associated in order to be active and that interaction among the enzymes in this system is enhanced when the enzyme solution is more concentrated.

TABLE II: Effects of Repeated Chromatography on the Activity of the ACP-Dependent Fatty Acid Synthetase.[a]

	Fractions 20–26	Pooled and Pptd Fractions 20–26	% ACP Dependency
First chromatography	299	990	99
Rechromatography	14	347	98

[a] All of the values given except the percentages represent total units of fatty acid synthetase activity as measured by the radioisotope assay described in the text. From greened cells, 0–70% saturation ammonium sulfate fractions were prepared and chromatographed on Bio-Gel A-15 as described in the text. Following assaying for enzyme activity, fractions 20–26 were pooled, precipitated with ammonium sulfate, dissolved in buffer I, and rechromatographed on the same column and treated as before.

The relationship between the initial reaction velocity and the amount of ACP-dependent enzyme was linear. Identical results were obtained with the radioactive assay and the spectrophotometric assay. The activity of the ACP-dependent enzyme system purified through Bio-Gel chromatography showed excellent agreement between the two assay methods. The velocity of oxidation of NADPH by a particular enzyme preparation was twice the rate of incorporation of [2-^{14}C]-malonyl-CoA into fatty acids. It should be recalled that 2 nmol of NADPH are oxidized during the incorporation of 1 nmol of malonyl-CoA into fatty acid. In addition, both reactions were linear in rate for 20 min.

When the spectrophotometric assay was used, the background rate of oxidation of NADPH was determined with each enzyme preparation and concentration used. Background was consistently low at about 0.005-absorbance unit decrease/min, whereas, in the presence of all substrates, the change was generally at least 0.05 absorbance unit/min. Nevertheless, correction for the background change in absorbance was made in all instances.

The optimum concentration was determined for all of the substances required by the ACP-dependent fatty acid synthetase. Figure 2 shows the relationship between the initial reaction velocities and the concentrations of acetyl-CoA, malonyl-CoA, and NADPH. Malonyl-CoA exhibits a typical hyperbolic curve of dependence of reaction velocity on substrate concentration. Using a Lineweaver–Burk plot of these data, a K_m of 1.4×10^{-5} M was calculated for malonyl-CoA. NADPH concentration dependence also describes a rectangular hyperbola, and a K_m for this substrate of 3.3×10^{-5} M was calculated.

Unlike the other two substrates, acetyl-CoA inhibits at high concentrations (Figure 2B). A concentration of 2×10^{-5} M appears to be optimal for enzyme activity under the conditions used. It must be stressed that all preparations of malonyl-CoA examined were contaminated to some extent with acetyl-CoA; the samples included those obtained commercially, both radioactively labeled and cold, as well as those synthesized and purified (Delo *et al.*, 1971) in our laboratory. Thin-layer chromatography on cellulose (Eastman Chromogram Sheet with fluorescent indicator) in butanol–acetic acid–water (50:20:30, v/v) of all preparations of malonyl-CoA indicated the presence of material chromatographing identically with acetyl-CoA, R_F 0.630. Malonyl-CoA in this system had an R_F of 0.413. When the source of the material chromatographed was [2-^{14}C]malonyl-CoA, the contaminating acetyl-CoA was radioactively labeled and was shown to represent 8% of the total material. The amounts of acetyl-CoA contaminating the malonyl-CoA preparations were sufficient to act as primers for fatty acid biosynthesis and were removed by preparative thin-layer chromatography.

The relationship of ACP concentration to the initial velocity of the reaction is complex. During the course of experimentation, it became evident that the initial reaction velocities varied depending on how the reaction was initiated. A series of reactions was done at saturating concentrations of ACP (9.05×10^{-6} M). In all instances, a 10-min incubation at 35° of the components was done prior to starting the reaction. When the reaction components, including enzyme and ACP, were incubated together and the reaction was initiated by the addition of malonyl-CoA, the greatest initial velocity was obtained. When the order of addition of enzyme, ACP, and

malonyl-CoA was varied, the initial reaction velocity was lower. When ACP and all other components were mixed and the reaction was started by adding enzyme which had been incubated separately at 35°, the initial velocity was about 90% of the maximum initial velocity. Finally when ACP was kept separate from the other reactants during the incubation period, the initial rate of reaction was about 70% of the maximum rate.

An examination of the relationship between ACP concentration and the initial reaction rates under the various conditions showed that the kinetic picture in each instance is different. Curves A and B of Figure 3 were obtained with enzyme aliquots from the same preparation. Portions of the enzyme were stored at −20° until used to determine two or three points, then discarded. Thus relatively freshly thawed enzyme was used in each determination. The data in curve C were obtained from another preparation of enzyme. In this instance, single aliquots of enzyme solution were thawed and used to determine each point. The described precautions were necessary since the enzyme gradually loses activity at 4° while it is stable at −20°.

When the enzyme system was incubated in the presence of ACP and all other substrates except malonyl-CoA (Figure 3A), the highest V_{max} was obtained, and the relationship between the initial rate and ACP concentration did not describe a rectangular hyperbola. A sigmoidal relationship between velocity and ACP concentration is seen under these conditions. A Lineweaver–Burk plot of these data was not linear.

When ACP was used to initiate the reaction, the relationship between initial reaction velocity and ACP concentration was as shown in Figure 3B. The initial rates at lower concentrations of substrate were faster than those in experiment A, and at higher concentrations, the rates were slower. The K_m for ACP under these conditions was 1.4×10^{-4} M.

Finally, when all of the components except enzyme were incubated together and reaction was initiated with enzyme which had been incubated separately, the kinetic data shown in Figure 3C were obtained. The curve resembles a rectangular hyperbola, and the data describe a linear Lineweaver–Burk plot. The K_m for ACP determined from these data was 1.1×10^{-6} M. A different preparation of enzyme was used in these experiments so the individual points in curve C cannot be compared directly to those in A and B. However, the K_m's and the general shapes of the curves are comparable.

Product Analysis. Thin-layer chromatography of the saponified reaction products indicated that the radioactivity was associated with a compound having an R_F 0.36. This result was obtained for both the ACP-dependent and the ACP-independent fatty acid synthetase systems. The R_F of a mixture of standards (myristic acid, palmitic acid, stearic acid, and oleic acid) was identical to that of the unknown products. That the product represented *de novo* fatty acid biosynthesis was shown when the reaction was carried out in the presence of [2-^{3}H]acetyl-CoA and [2-^{14}C]malonyl-CoA. The ^{3}H incorporation marks the methyl end of a new fatty acid chain. The ratio of incorporation of ^{14}C to ^{3}H (11.28 nmol/1.46 nmol) is an estimation of the chain length of the fatty acids made by the enzyme system which was seen to be about 18 carbon atoms. Delo *et al.* (1971) found that greater than 60% of the product made by a similar enzyme system from *Euglena gracilis* strain Z was a fatty acid of 18-carbon chain length. The presence of an acetyl-CoA carboxylase activity leading to incorporation of ^{3}H in the form of [^{3}H]malonyl-CoA was ruled out experimentally.

An unsaponified reaction solution was chromatographed on a double layer Bio-Gel column. Bio-Gel of two different exclusion ranges was used in order to separate the macromolecules, *i.e.*, ACP and the synthetase enzymes, as well as the smaller molecules. In the effluent diagram shown in Figure 4, the largest peak of radioactivity represents the original substrate, [2-^{14}C]malonyl-CoA; this appears immediately behind the bacitracin marker. The radioactive peak which appeared between the second (cytochrome *c*) and third (bacitracin) markers most likely represents product-bound ACP. The third radioactive peak which appears as a shoulder to the largest peak is taken to be free product and trailing [2-^{14}C]-

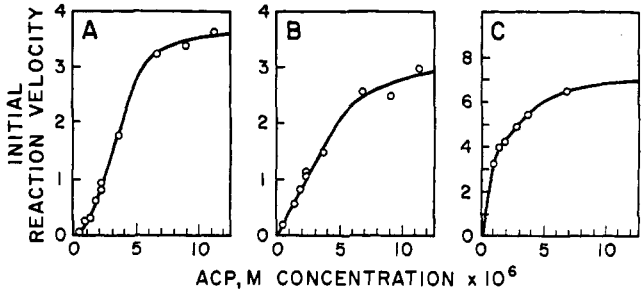

FIGURE 3: Effects of ACP concentration and order of addition of the initial reaction velocity of the ACP-dependent fatty acid synthetase. The experiments were carried out as described in the text. The same enzyme preparation was used in determining the points shown in A and B; a different but similarly prepared enzyme was used for C. The data in A were obtained from experiments where reaction was initiated by the addition of malonyl-CoA. B experiments were initiated by the addition of ACP. C experiments were initiated by the addition of enzyme. In all instances, all components except the initiation one were incubated together at 35° for 10 min prior to initiation. The substances used to start the reactions were incubated separately at 35°. Fatty acid biosynthesis was assayed spectrophotometrically.

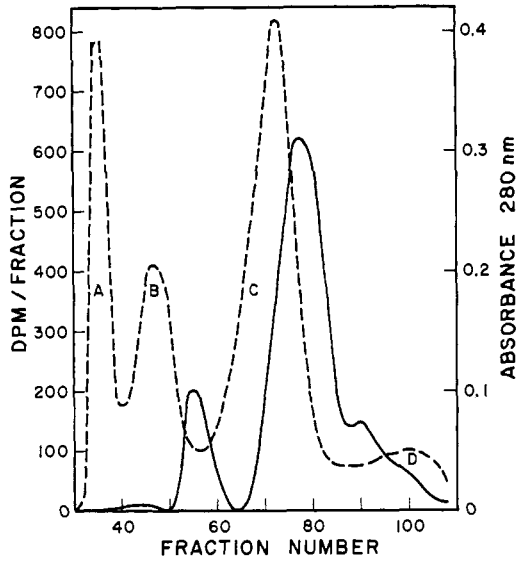

FIGURE 4: Chromatographic separation of the products of the ACP-dependent fatty acid synthetase. A radioactive assay solution prepared as described in the text was applied to a double-layer Bio-Gel column (Bio-Gel P-100, 2.5 × 10.3 cm, and Bio-Gel P-2, 2.5 × 8.7 cm) equilibrated in and eluted with 0.05 M acetate buffer pH 5.0. Fractions of 1.0 ml were collected, and 0.1-ml aliquots were measured for radioactivity. A mixture of calibration standards containing ferritin, horse heart cytochrome *c*, bacitracin, tyrosine, and dichlorofluorescein in 0.5 ml was run under the same conditions. These are represented by peaks A, B, C, and D respectively. Absorbance of standards at 280 nm (- - - -); radioactivity (——).

malonic acid from hydrolysis of malonyl-CoA. Quantitation of this experiment is difficult because of partial hydrolysis of the thiolesters. The extent of hydrolysis of the thiolesters which may have occurred during the assay and chromatography can be estimated roughly from the peak of unreacted substrate; the measured peak contained about 7500 dpm. A total of 28,600 dpm of substrate had been added originally, and 11,800 dpm was measured as incorporated into substrate in a parallel assay. This would leave 16,800 dpm of unreacted substrate; the column yielded 7500 dpm in this peak area suggesting that about 55% of the malonyl-CoA had hydrolyzed. In the chromatogram, approximately 2700 dpm is seen in the first peak of radioactivity, the location of which is compatible with acyl-ACP. If 55% of this substance had been hydrolyzed during the experiment, the original activity here would have been about 4900 dpm which represents 60% of the total activity (8160 dpm) recovered from the column which could be designated as free fatty acids or ACP-bound fatty acids. Thus a substantial proportion of the products formed by the ACP-dependent fatty acid synthetase is bound to ACP.

These results were quantitated in another experiment in which the free fatty acids were extracted with pentane from a chilled acidified assay solution immediately upon cessation of reaction. The amount of free product was very little, only 2% of the total [2-^{14}C]malonic acid incorporated. Following the initial pentane extractions, the reaction solution was saponified at 100° for 30 min. After acidification of the saponified solution, additional pentane extractions were done which yielded 8.638 nmol of [2-^{14}C]malonic acid incorporated into product. Again, the major portion of the product of the ACP-dependent fatty acid synthetase is shown to exist in a bound form, compatible with acyl-ACP.

Discussion

A difference in the patterns of fatty acid synthetase systems dependent on the developmental stage of *Euglena gracilis* variety *bacillarius* does exist. Organisms which were kept in darkness yield relatively little of the ACP-dependent enzyme system, whereas in greened organisms the ACP-dependent and ACP-independent enzyme activities each comprised about 50% of the total fatty acid synthetase activity obtained from greened cells. However, the total fatty acid synthetase activity tends to remain constant regardless of the physiologic state of the cells and regardless of which system is comprising this activity. Clearly, there is a striking change in both of the enzyme systems in response to the transition of the cells from darkness to light. In general, this is similar to the response to the same change in conditions reported for *Euglena gracilis* strain Z (Ernst-Fonberg and Bloch, 1971)

In addition to the evidence provided by ACP dependency, the chromatography experiments indicate that the ACP-dependent fatty acid synthetase is composed of discrete proteins which are loosely associated relative to the ACP-independent multienzyme complex. Although repeated chromatography on Bio-Gel results in a lower proportion of the total activity present appearing as a discrete peak of activity, indicating relatively weak interactions, it appears that there is a tendency for this system to associate loosely in solution. After the first chromatography, a relatively large peak of enzyme activity is seen corresponding to a molecular weight of about 360,000. This represents a molecular weight where all the enzymes constituting the ACP-dependent fatty acid synthetase are present. It is likely that the molecular weights of the individual enzymes are so large that this is the position in which they are chromatographing as overlapping peaks of discrete dissociated proteins. The molecular weights of some of the individual enzymes comprising the *E. coli* fatty acid synthetase have been determined to be below 100,000 (Joshi and Wakil, 1971; Greenspan *et al.*, 1969). Also when Sumper *et al.* (1969) dissociated the yeast fatty acid synthetase complex into inactive subunits of 200,000–250,000 molecular weight, they questioned whether the dissociation resulted in single component enzymes.

The immediate products of the reaction catalyzed by the ACP-dependent fatty acid synthetase are not free fatty acids but are bound, presumably to ACP. This is similar to the situation seen in the *E. coli* fatty acid synthetase where a specific palmityl thioesterase has been described by Barnes and Wakil (1968). Acyl-ACP is involved in reactions other than fatty acid biosynthesis (Ailhaud and Vagelos, 1967; Vagelos *et al.*, 1966; Kass and Bloch, 1967; Nagai and Bloch, 1966) so it is plausible that the acyl-ACP product is a suitable substrate for synthesis of complex lipids.

Malonyl-CoA does not inhibit the ACP-dependent fatty acid synthetase within the range measured. The synthetase complex from pigeon liver is extremely sensitive to inhibition by malonyl-CoA (Plate *et al.*, 1968), whereas the enzyme system obtained from rat mammary gland is not inhibited (Smith and Abraham, 1970). The K_m of 1.4×10^{-5} M for malonyl-CoA in the *Euglena* ACP-dependent system is comparable to the values of 1.3×10^{-5} M for the enzyme systems from rat mammary gland and pigeon liver. It is also comparable to the value of 5.4×10^{-5} M for the malonyl transacylase of *E. coli* (Joshi and Wakil, 1971).

Acetyl-CoA above optimum concentration exerts an inhibitory effect on the ACP-dependent fatty acid synthetase. A similar observation was made for the enzyme system from pigeon liver (Plate *et al.*, 1968) but was not observed with the enzyme from rat mammary gland (Smith and Abraham, 1970). The acetyl-CoA concentration where the maximum rate in the *Euglena* system was seen corresponds with the range of K_m values, from 0.2×10^{-5} to 2.2×10^{-5} M, reported for this substrate from the other systems cited.

The K_m for NADPH under the described conditions was calculated to be 3.3×10^{-5} M. Again, this is comparable to the corresponding K_m's of 3.4×10^{-5} and 0.9×10^{-5} M reported for the rat liver and pigeon liver systems cited above.

These data indicate similarities in kinetic parameters obtained for fatty acid synthetases which are isolated as strongly associated multienzyme complexes with ACP tightly bound and a fatty acid synthetase which is not isolated as a multienzyme complex. The latter system is dependent on added ACP for activity following partial purification and would seem to fit the criteria of the type II fatty acid synthetases described by Brindley and coworkers (1969). The similarities do not prove but are compatible with the possibility that the ACP-dependent fatty acid synthetase system functions in solution as a highly organized complex of enzymes. Results which may be interpreted to support this possibility are seen in the effects of the order of addition and the concentration of ACP on the initial reaction velocity. In experiments where ACP and the *Euglena* fatty acid synthetase enzymes are incubated together prior to initiation of reaction by malonyl-CoA, a sigmoid curve and the highest V_{max} are obtained. This may indicate that ACP plays a role in organizing the enzymes of the ACP-dependent fatty acid synthetase. Under conditions where enzyme and ACP are not incubated to-

gether prior to initiation of reaction, the values for V_{max} are less.

This situation may represent a means of cellular control of fatty acid synthetase activity. Clearly the rate of activity is very sensitive to ACP concentration, and the sigmoid curve of response to ACP concentration can be imagined to provide the greatest regulatory latitude. Control of the level of one protein provides a means of regulating the activities of at least six other proteins. The possibility that the enzymic response to ACP concentration may represent a control mechanism is supported by the fact that the cellular concentration of ACP is rigidly controlled in *E. coli* (Alberts and Vagelos, 1966). Also, Vagelos and Larrabee (1967) have suggested a regulatory role in metabolism for ACP levels in conjunction with their studies of a highly specific ACP hydrolase.

The data presented here indicate that a great deal of the complexity of the fatty acid synthetase multienzyme complexes can be seen in a system which is readily dissociated and is dependent *in vitro* on exogenous ACP. Possibly the problems posed by the multienzyme complexes are more amenable to study in this system where dissociation into components need not be dependent on relatively harsh methods and where ready association into an active complex clearly occurs. Also, the likelihood that ACP may play a role in enhancing this association of enzymes is interesting.

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